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ORIGINAL ARTICLE ***Reproductive biology***

SIRT1 signalling protects mouse oocytes against oxidative stress and is deregulated during aging

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STUDY QUESTION: Is SIRT1 involved in the oxidative stress (OS) response in mouse oocytes?

SUMMARY ANSWER: SIRT1 plays a pivotal role in the adaptive response of mouse germinal vesicle (GV) oocytes to OS and promotes a signalling cascade leading to up-regulation of the MnSod gene.

WHAT IS KNOWN ALREADY: OS is known to continuously threaten acquisition and maintenance of oocyte developmental potential during *in vivo* processes and *in vitro* manipulations. Previous studies in somatic cells have provided strong evidence for the role of SIRT1 as a sensor of the cell redox state and a protector against OS and aging.

STUDY DESIGN, SIZE, DURATION: GV oocytes obtained from young (4–8 weeks) and reproductively old (48–52 weeks) CD1 mice were blocked in the prophase stage by 0.5 μM cilostamide. Groups of 30 oocytes were exposed to 25 μM H_2O_2 and processed following different times for the analysis of intracellular localization of SIRT1 and FOXO3A, and evaluation of Sirt1, miRNA-132, FoxO3a and MnSod gene expression. Another set of oocytes was cultured in the presence or absence of the SIRT1-specific inhibitor Ex527, and exposed to H_2O_2 in order to assess the involvement of SIRT1 in the activation of a FoxO3a-MnSod axis and ROS detoxification. In the last part of this study, GV oocytes were maturated *in vitro* in the presence of different Ex527 concentrations (0, 2.5, 5, 10, 20 μM) and assessed for maturation rates following 16 h. Effects of Ex527 on spindle morphology and ROS levels were also evaluated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: SIRT1 and FOXO3A intracellular distribution in response to OS was investigated by immunocytochemistry. Real-time RT–PCR was employed to analyse Sirt1, miR-132, FoxO3a and MnSod gene expression. Reactive oxygen species (ROS) production was evaluated by *in vivo* measurement of carboxy-H_2DCF diacetate labelling. Spindle and chromosomal distribution in *in vitro* matured oocytes were analysed by immunocytochemistry and DNA fluorescent labelling, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: Specific changes in the intracellular localization of SIRT1 and up-regulation of Sirt1 gene were detected in mouse oocytes in response to OS. Moreover, increased intracellular ROS were observed when SIRT1 activity was inhibited by Ex527. In aged oocytes Sirt1 was expressed more than in young oocytes but SIRT1 protein was undetectable. Upon OS, significant changes in miR-132 micro-RNA, a validated Sirt1 modulator, were observed. A negative correlation between Sirt1 mRNA and miR-132 levels was observed when young oocytes exposed to OS were compared with young control oocytes, and when aged oocytes were compared with young control oocytes. FoxO3a and MnSod transcripts were increased upon OS with the same kinetics as Sirt1 transcripts, and up-regulation of MnSod gene was prevented by oocyte treatment with Ex527, indicating that SIRT1 acts upstream to the FoxO3a-MnSod axis. Finally, the results of the *in vitro* maturation assay suggested that SIRT1 might be involved in oocyte maturation by regulating the redox state and ensuring normal spindle assembly.

LIMITATIONS, REASONS FOR CAUTION: The main limitation of this study was the absence of direct quantification of SIRT1 enzymatic activity due to the lack of an appropriately sensitive method.

WIDER IMPLICATIONS OF THE FINDINGS: The present findings may provide a valuable background for studying the regulation of SIRT1 during oogenesis and its relevance as a sensor of oocyte redox state and energy status. The antioxidant response orchestrated by SIRT1 in oocytes

seems to decrease with aging. This suggests that SIRT1 could be an excellent pharmacological target for improving oocyte quality and IVF outcome in aging or aging-like diseases.

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Key words: sirtuins / SIRT1 / oxidative stress / oocyte aging / miRNA

Introduction

Human female reproductive function reaches a maximum efficiency in early 20s and then decreases in a manner that is universal through mammalian species (Wood, 1989). The gradual loss of fertility becomes more marked in the late 30s ending in menopause at a mean age of 50–51 years (te Velde and Pearson, 2002). The main regulator of female reproductive function is the ovary. Its primary role is to provide a reserve of germ cells established prior to or shortly after birth, which gradually decreases in quality and quantity during the reproductive lifespan. At puberty the gonad is endowed with 300 000 primordial follicles containing oocytes that are blocked at prophase of the first meiotic division and can be recruited into the growing phase until menopause occurs. Most of them become atretic while just a few reach the large antral follicle stage and complete meiosis to the metaphase II stage (MII) during ovulation to become competent for fertilization (Hennet and Combelles, 2012; Sánchez and Smitz, 2012; Sobinoff *et al.*, 2013).

A gradual accumulation of damage by free radicals and dicarbonyl reactive compounds along with a reduced detoxifying capacity has been considered the major mechanisms underlying ovarian aging (Li *et al.*, 2012; Meldrum, 2013; Tatone and Amicarelli, 2013). The increase in aneuploidy represents the most evident effect of the unhealthy status of oocytes produced in advanced reproductive age (the so-called aged oocytes) as a result of prolonged exposure of follicles to factors which can irreversibly accumulate during the reproductive lifespan in the ovarian and follicle microenvironment (Tatone *et al.*, 2008). Spindle aberrations, disturbances in chromosome congression, mitochondrial alterations and changes in gene and protein expression represent further characteristics of the aged oocyte (Eichenlaub-Ritter, 2012).

Although the oocyte aging phenotype has been well characterized, the adaptive response machinery to oxidative stress (OS) has been so far poorly investigated. Recently, a growing body of evidence supports the hypothesis that the aging process is regulated by a continuous cross-talk between reactive oxygen species (ROS) and SIRT1, the most extensively studied sirtuin. Sirtuins (silent information regulator 2 (Sir2) proteins) belong to a well-conserved family of NAD^+-dependent enzymes with deacetylase and/or mono-ADP-ribosyl transferase activity. The seven members of the mammalian sirtuin family have evolved to respond to a variety of stresses and are emerging as key anti-aging molecules and regulators in many diseases (Michan and Sinclair, 2007; Morris, 2013). SIRT1 has been shown to be the key molecule responsible for extending lifespan in response to caloric restriction (Cohen *et al.*, 2004; Rogina and Helfand, 2004). Through deacetylation of stress responsive transcription factors, co-regulators and enzymes, SIRT1 is involved in metabolic control at cellular, tissue and whole-body levels (Schwer and Verdin, 2008). Since NAD^+ is its unique co-substrate, SIRT1 acts as a sensor of the intracellular energy status and as a redox-sensitive deacetylase as well (Tanner *et al.*, 2000; Houtkooper *et al.*, 2010). Through deacetylation of the FOXO3A transcription factor, SIRT1 stimulates the expression of catalase and manganese superoxide dismutase (MnSOD) thereby promoting cell survival under oxidant conditions (Caito *et al.*, 2010). Accordingly, overexpression of mouse SIRT1 has been reported to increase cell survival after exposure to hydrogen peroxide (Luo *et al.*, 2001).

In addition to the availability of NAD^+ and substrates, the activity of SIRT1 can be fine-tuned by a variety of modulators including its own expression levels that are under the influence of several miRNAs known to suppress Sirt1 mRNA translation or reduce its stability (Yamakuchi, 2012; Chen *et al.*, 2013; Revollo and Li, 2013).

Recently, a possible role of SIRT1 in the regulation of fertility has emerged. Reproductive defects in both sexes have been described in SIRT1-null animals that exhibit sterility or altered gametogenesis and offspring with reduced vitality (McBurney *et al.*, 2003; Coussens *et al.*, 2008). The expression of all sirtuins has been observed in mouse oocytes and embryos. Among them SIRT3 seems to be involved in protecting against stress conditions during *in vitro* fertilization and culture (Kawamura *et al.*, 2010). A recent study, focused on prevention of aging by dietary antioxidant strategies, has provided indirect evidence for a crucial role of SIRT1 activity in the regulation of the ovarian aging process (Liu *et al.*, 2012).

Based on the above observations, the aim of the present study was to investigate the involvement of SIRT1 signalling in protecting ovarian fully-grown oocytes against OS and to identify possible alterations linked to ovarian aging.

Materials and Methods

Oocyte collection

Oocytes were isolated from CD-1 mice (Charles River s.r.l. Calco, Italy) at the age of 4–8 weeks (young mice) and 48–52 weeks (reproductively old mice). All the experiments were carried out in accordance with the guidelines for the care and use of laboratory animals approved by the Animal Care Committee of the University of L'Aquila. Ovarian immature oocytes at the germinal vesicle stage (GV) were collected after vaginal smears analysis to identify females in diestrous of the spontaneous oestrous cycle as described previously (Vogt *et al.*, 2009). A meiotic block at GV stage was achieved by addition of 0.5 μM cilostamide (Sigma-Aldrich, St. Louis, MO, USA) (IC_{50} in mouse oocytes, 0.1 μM, Wiersma *et al.*, 1998) in the culture media, M2 or M16 (Sigma-Aldrich), according to each procedure. In order to obtain mature oocytes, females were superovulated by intraperitoneal injection of 7.5 IU of PMSG (Folligon; Intervet-International, Boxmeer, Holland) and 7.5 IU of hCG (Profasi HP 2000; Serono, Roma, Italy) 48 h apart. After 15 h oviducts were removed and oocytes arrested at metaphase II stage (MII) were isolated after a brief exposure to 0.3 mg/ml hyaluronidase (Sigma-Aldrich). Degenerated or fragmented cells were discarded and healthy oocytes were pooled and randomized before distribution into the experimental groups. Each experiment was performed three times and at least 30 oocytes per group were employed in each replicate.

Oxidative stress treatments

To identify the proper stimulus to induce OS in GV oocytes, oocytes were exposed to different H_2O_2 treatments: 10 mM H_2O_2 for 2 min (Bogliolo *et al.*, 2013), 100 μM H_2O_2 for 10 min (Takahashi *et al.*, 2003) or 25 μM H_2O_2 for 30 min (Choi *et al.*, 2007). GV oocytes were then extensively washed and incubated at 37°C and 5% CO_2 in air for 24 h. Oocytes were checked at 16 h and 24 h for polar body emission and degeneration rate, respectively.

Oocyte treatments

To induce OS, GV-blocked oocytes were exposed to 25 μM H_2O_2 for 30 min and then cultured for cell recovery for 30 min, 60 min, 90 min, 120 min or 180 min at 37°C, 5% CO_2 in air in M16 medium. In all of the experiments, control untreated GV oocytes were processed soon after retrieval. To obtain inhibition of SIRT1 activity in the study of ROS levels and MnSod gene expression, GV-arrested oocytes were placed in M16 medium in the presence of 20 μM Ex527 (Sigma-Aldrich) (Schuster *et al.*, 2014), a SIRT1-specific inhibitor, for 1 h at 37°C, 5% CO_2 in air. Ex527-exposed oocytes were then stressed by 25 μM H_2O_2 for 30 min and cultured for 90 min at 37°C, 5% CO_2 in air in M16 medium containing 20 μM Ex527. Oocytes maintained in M16 medium for 1 h were exposed to H_2O_2 and then cultured for 90 min. A group of oocytes were exposed to Ex527 for 1 h prior to be processed for MnSod analysis. To study the effects of Ex527 on *in vitro* maturation (IVM), young GV oocytes were placed into M16 medium in the presence of Ex527 concentrations ranging from 2.5 to 20 μM and then cultured for 16 h at 37°C and 5% CO_2 in air. Some of the oocytes were incubated with 500 μM *N*-acetyl-L-cysteine (NAC, Sigma-Aldrich) in the presence or absence of 20 μM Ex527. Control oocytes were matured in the presence of 0.001% DMSO (Sigma-Aldrich) to evaluate a possible toxic effect of DMSO in which Ex527 was dissolved. Numbers of oocytes that emitted first polar body (MII), oocytes that had resumed meiosis (GVBD, germinal vesicle breakdown), immature oocytes and degenerated oocytes were recorded.

Immunofluorescence and confocal analysis

Immunofluorescence experiments were performed according to Tatone *et al.* (2011). After removal of zona pellucida by Tyrode's acid solution (Sigma-Aldrich), oocytes were fixed for immunofluorescence and labelled by first antibody [rabbit anti-SIRT1 (Abcam, Cambridge, UK), rabbit anti-FOXO3A (Abcam) or mouse anti-α-tubulin (Sigma-Aldrich)] and second antibody [anti-rabbit-Alexa 488 (Life Technologies, Carlsbad, CA, USA) or anti mouse-FITC (Sigma-Aldrich)]. Chromatin staining was performed by propidium iodide (Sigma-Aldrich). In negative control oocytes, the primary antibody was omitted. Oocytes were mounted on slides and analysed under confocal microscope (TCS SP5 II, Leica Microsystems, Germany).

In vivo detection of intracellular ROS levels

ROS levels in oocytes were assessed according to Takahashi *et al.* (2009). This method is based on the use of a fluorescent dye, the 5-(and 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCF diacetate), which is a carboxylated analogue of H_2DCF diacetate. This compound is characterized by negative charges, which allow H_2DCF diacetate to pass through the plasmatic membrane and remain in the intracellular space for long periods. In the cell, H_2DCF diacetate can be oxidized with production of carboxydichlorofluorescein (carboxy-DCF), which is a fluorescent molecule and an indicator of ROS.

Oocytes from different conditions were processed at the same time. Oocytes were incubated for 30 min at 37°C in M2 supplemented with 10 μM carboxy-H_2DCF diacetate (Life Technologies). After washing, oocytes were mounted onto slide and observed under a fluorescence microscope (excitation at 480 nm, emission at 520 nm) with identical PMT settings. Images were analysed by ImageJ 1.44p software.

Real time RT–PCR analysis

All reagents used for Real-time RT–PCR were obtained from Life Technologies. Pools of 30 oocytes were washed in RNase free water, then transferred in 10 μL of RNase free water and sunk in liquid nitrogen. Samples were stored at −80°C until use. RNA extraction was performed by Picopure kit. Genomic DNA was degraded by DNA free kit (Applied Biosystem, Foster City, CA, USA).

To assess Sirt1, FoxO3a, MnSod and β-actin gene expression, reverse transcription of RNA was carried out by SuperScript VILO cDNA Synth Kit. Polymerase chain reaction (PCR) analyses were carried out in triplicate by Power SYBR Green PCR Master Mix kit (Applied Biosystem), according to manufacturer's instructions. Each reaction was performed in 25 μL containing the cDNA equivalent of 1.5 oocytes and β-actin was used as internal standard. Primer sequences are shown in Table I. For each reaction, 40 cycles of amplification with the following profile were performed: 95°C for 10 min for the first cycle; 95°C for 15 sec and 60°C for 1 min for 40 cycles; then 95°C for 15 s and 60°C for 15 s. Relative intensity of PCR-specific amplicons was calculated by ΔΔCt, obtained by normalization of the level of each transcript to that of β-actin and to the normalized level of transcript of the control group (Livak and Schmittgen, 2001).

For detection of miR-132, reverse transcription of RNA was carried out by TaqMan MicroRNA Reverse Transcription Kit. PCR analyses were carried out in triplicate by TaqMan MicroRNA Assay and TaqMan Gene Expression Master Mix (Applied Biosystem). For each reaction, 40 cycles of amplification with the following profile were performed: 95°C for 10 min for the first cycle; 95°C for 15 s and 60°C for 1 min for 40 cycles; then 95°C for 15 s and 60°C for 15 s.

To assess Sirt1 and Hprt1 genes, RNA was reverse transcribed by SuperScript VILO cDNA Synth Kit cDNA. Hprt1 was used as internal standard for both miR-132 and Sirt1 (Barbagallo *et al.*, 2013). The mean fold change is shown as natural logarithm of RQ values and the error is estimated by evaluating the $2^{-\Delta\Delta Ct}$ equation using ΔΔCt plus standard deviation and ΔΔCt minus the standard deviation. The correlation between miR-132 and Sirt1 was performed by Pearson correlation test.

Table I Primer sequences.

	Forward	Reverse
Sirt1	5′-GCAACAGCATCTTGCCTGAT-3′	5′-GTGCTACTGGTCTCACTT-3′
FoxO3a	5′-CGGCTCACTTTGTCCCAGAT-3′	5′-GCCGGATGGAGTTCTTCCA-3′
MnSod	5′-ATTAACGCGCAGATCATGCA-3′	5′-TGTCCCCCACCATTGAACTT-3′
β-Actin	5′-GAGACCTTCAACACCCCAGC-3′	5′-ATGTCACGCACGATTTCCC-3′
Hprt1	5′-TCAGTCAACGGGGGACATAAA-3′	5′-GGGGCTGTACTGCTTAACCAG-3′

Western blotting analysis

One hundred GV oocytes from young or reproductively aged mice were pooled in Laemli buffer (Sigma-Aldrich) and stored at −80°C. After thawing, samples were denatured at 95°C for 5 min and loaded on a 10% SDS–PAGE and blotted on polyvinyl fluoride (PVDF) membranes (Sigma-Aldrich). Non-specific binding sites were blocked overnight with 5% not fat dry milk (Bio-Rad Laboratories, Italy) in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were incubated with polyclonal rabbit anti-SIRT1 (Abcam) and rabbit anti-β-actin antibody (Abcam) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (Abcam) for 1 h at room temperature. After washing, specific immunoreactive complexes were detected by ECL kit (Thermo Scientific, Waltham, MA, USA) and Uvitec Cambridge system (Alliance series, Cambridge, UK). SIRT1 bands were normalized for β-actin using ImageJ 1.44p software and values were given as relative units (RU). The experiment was performed in triplicate.

Statistical analysis

For each experimental series, values are reported as mean ± SEM. Statistical analysis was assessed by One Way ANOVA followed by Student Keuls Multiple Comparison. Analyses were performed using the SigmaStat software (Jandel Scientific Corporation, San Rafael, CA, USA). A P-value of <0.05 was considered statistically significant.

Results

Intracellular localization of SIRT1 in response to oxidative stress

In the absence of a quantitative assay of redox potential in follicular fluid or previous studies reporting OS challenge in GV oocytes, we initially tested OS treatments in MII oocytes (Takahashi *et al.*, 2003; Choi *et al.*, 2007; Bogliolo *et al.*, 2013). Based on IVM results, we selected 25 μM H_2O_2 for 30 min as the proper dose and time of exposure to be employed in our study. In contrast to the other treatments, this time and dose slightly affected the IVM rate but did not compromise cell viability (Supplementary data, Table SI).

In the first set of experiments, we investigated the involvement of SIRT1 in GV oocyte response to OS by evaluating changes of intracellular localization. With this aim, oocytes were exposed to 25 μM H_2O_2 for 30 min and processed for immunocytochemistry immediately or after a 30 min recovery in M16 medium. As shown in Fig. 1a-a'', control

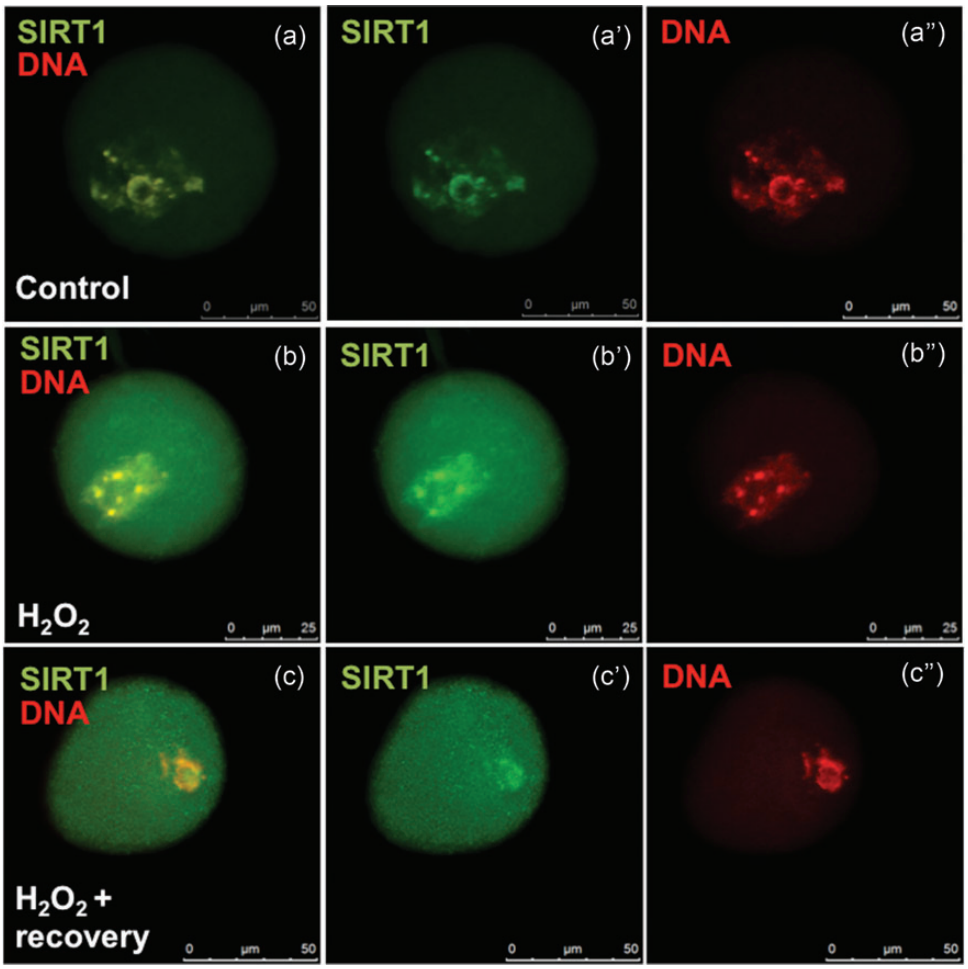

Figure 1 Changes of intracellular localization of SIRT1 after oxidative stress. Micrographs of representative Sirt1 localization in control untreated germinal vesicle (GV) oocytes (**a**–**a**''), in oocytes exposed to H_2O_2 for 30 min during GV-stage arrest by cilostamide (**b**–**b**'') and oocytes exposed H_2O_2 for 30 min and cultured to recover for 30 min (**c**–**c**''). These staining patterns were presented in >80% of oocytes analysed in each group.

Table II Effects of a specific SIRT1 inhibitor on reactive oxygen species (ROS) levels in GV oocytes‡.

	No recovery		90 min after stress	
	n	Mean pixel intensity (± SEM)	*n*	Mean pixel intensity (± SEM)
Control oocytes	91	20 186 (± 10 426)*,a	89	23 136 (± 15 284)*,a
25 μM H_2O_2	96	33 656 (± 15 355)*,b	91	22 947 (± 13 734)*,a
20 μM Ex527 + 25 μM H_2O_2	93	32 086 (± 21 993)*,b	92	37 022 (± 20 455)*,b

‡Analysis of ROS production in oocytes exposed to H_2O_2 for 30 min, cultured in presence or absence of SIRT1-specific inhibitor, Ex 527, soon after oxidative stress or after 90 min of recovery. Significant differences among the groups by one-way ANOVA: *$P < 0.001$; multiple pairwise comparison by Student Newman Keuls test: a, b$P < 0.05$.

oocytes exhibited a prevalent nuclear localization where SIRT1 fluorescence appeared overlapping with stained DNA. Immediately after H_2O_2 exposure (Fig. 1b-b''), intense cytoplasmic staining of SIRT1 was observed in the presence of increased fluorescence in the nucleus. In oocytes analysed following 30 min (Fig. 1c-c'') exposure, an intense fluorescence was maintained but the merged signals revealed a reduced overlap between SIRT1 and DNA labelling.

Ability of SIRT1 to regulate ROS levels during oxidative stress

To establish whether SIRT1 plays an active role in protection against OS, young GV oocytes were exposed to 20 μM Ex527, a SIRT1-specific inhibitor, for 1 h prior to H_2O_2 exposure. ROS levels were analysed immediately (time 0) or 90 min after stress as described in the Materials and Methods. As expected (Table II), oocytes analysed immediately after H_2O_2 exposure showed increased ROS labelling when compared with control untreated oocytes. Then ROS levels decreased to values similar to that observed in control oocytes at 90 min after the stress. When oocytes were exposed to 20 μM Ex527, the ROS labelling observed at time 0 was not significantly different from the H_2O_2 group. However at 90 min after the stress, ROS levels were significantly higher than those monitored in the absence of the inhibitor.

Modulation of Sirt1 expression during oxidative stress response and aging

Changes in the expression of the Sirt1 gene in response to oxidative stress

In order to investigate whether Sirt1 is up-regulated upon OS, we monitored mRNA levels in GV oocytes stressed with H_2O_2 and processed for real-time PCR after 60, 90, 120 or 180 min. The results revealed that OS induced a significant increase in Sirt1 mRNA levels detectable at any time analysed (Fig. 2) with a peak recorded after 90 min when a 7-fold increase in Sirt1 levels was observed.

Changes in the expression of Sirt1 during aging

We also monitored Sirt1 expression in mouse oocytes from reproductively old mice. Results from real-time RT–PCR revealed higher levels of Sirt1 mRNA in aged oocytes. Nevertheless, at 90 min after OS, Sirt1 mRNA expression was maintained at the same level as in aged untreated oocytes (Fig. 3A). Furthermore, when expression at the protein level was analysed, SIRT1 appeared undetectable in the aged oocytes (Fig. 3B).

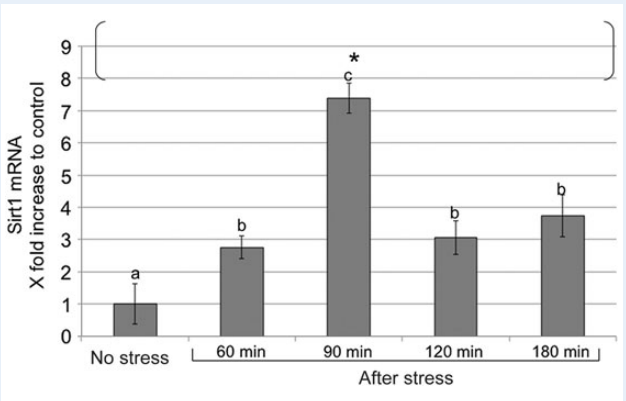

Figure 2 Changes in gene expression of Sirt1 in oocytes stressed by H_2O_2. Analysis of Sirt1 expression in oocytes stressed by H_2O_2 and recovered for 60, 90, 120 or 180 min. Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.001$, one-way ANOVA; a, b, c: $P < 0.05$, Student Newman Keuls test.

Changes in miRNA-132 levels in young and aged oocytes in relation to oxidative stress and Sirt1 expression

In order to identify a possible post-transcriptional modulation of Sirt1, we monitored the levels of miR-132 levels, a validated Sirt1 modulator (Strum *et al.*, 2009), in young and aged oocytes in response to stress. As shown in Fig. 4A, miR-132 levels in young oocytes decreased significantly with OS, reaching levels similar to those observed in untreated aged oocytes. Moreover, in contrast to young oocytes, no change in miR-132 levels was detected with OS in aged oocytes. When changes in both Sirt1 and miR-132 were analysed in the same sample, we observed a negative correlation between Sirt1 mRNA and miR-132 that was statistically significant when young oocytes exposed to OS were compared with young control oocytes, or when aged oocytes were compared with young control oocytes (Fig. 4B).

Sirt1-related pathway in response to oxidative stress

Given the central role of FOXO3A in the activation of adaptive response to OS, we monitored changes of intracellular localization of this transcription factor. As shown in Fig. 5, control oocytes exhibited a homogenous fluorescence that in most oocytes was associated with the

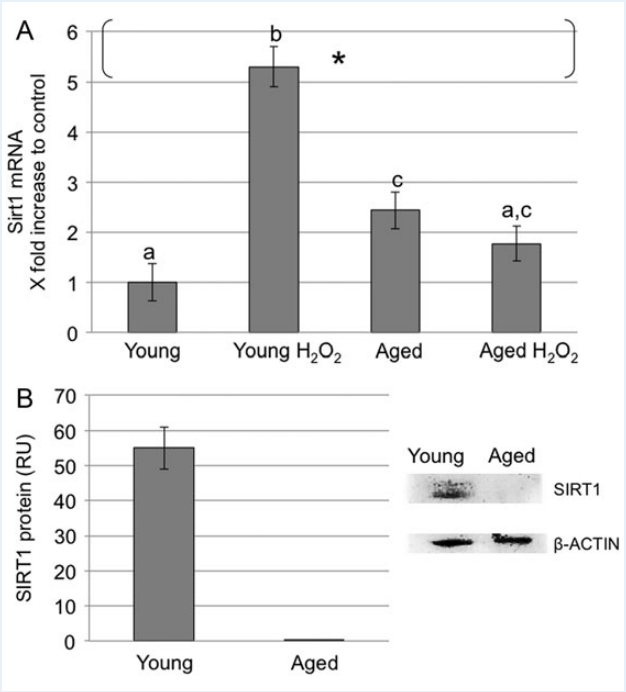

Figure 3 Effects of aging on Sirt1 expression. (**A**) Histogram showing Sirt1 mRNA levels in young and aged oocytes. Oocytes exposed to OS were analysed 90 min after H_2O_2 exposure. Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.001$, one-way ANOVA; a, b, c: $P < 0.05$, Student Newman Keuls test. (**B**) Histogram of densitometric analysis of SIRT1 protein in young and aged oocytes. As shown, in young oocytes the SIRT1 immunoreactive band is evident; in aged oocytes it is undetectable. β-actin is used here as the internal standard.

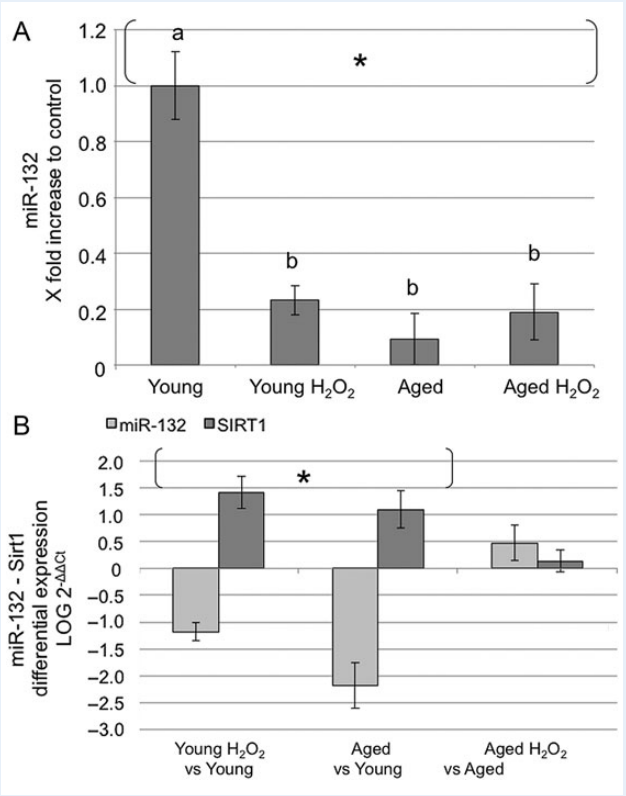

Figure 4 Effects of oxidative stress on miR-132 levels in young and aged oocytes. (**A**) Histogram showing miR-132 levels in young and aged oocytes in response to OS, analysed 90 min after H_2O_2 treatment. Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.001$, one-way ANOVA; a, b: $P < 0.05$, Student Newman Keuls test. (**B**) Sirt1 and miR-132 differential expression in young and aged oocytes in response to OS. Values are means ± SEM of three determinations. The fold change of each gene is shown as ln $2^{-\Delta\Delta Ct}$. Statistical comparisons by Pearson correlation reveals an anticorrelation between the two products in stressed and aged oocytes when compared with young controls. *:$P = 0.98$, Pearson correlation.

presence of fluorescent spots in the nuclear region. Immediately after H_2O_2 exposure, intense nuclear staining was observed with a reduction of fluorescence in the cytoplasm. In oocytes analysed after 30 min recovery, an increase in total fluorescence was detected.

In subsequent experiments changes in the expression of FoxO3a and MnSod genes were analysed in order to identify the role of the OS in the induction of their transcription. To this end, we monitored mRNA levels of these transcripts in GV oocytes at different times after stress. The results revealed that OS induced a significant increase in both FoxO3a and MnSod mRNA levels at 90 min followed by a decline at 120 min (Fig. 6).

To establish the role of SIRT1 in the activation of the response to OS, we investigated the effects of Ex527 on the expression of MnSod gene. Real-time RT–PCR analysis revealed that the increase in MnSod mRNA levels upon stress was prevented by oocyte exposure to Ex 527 (Fig. 7).

In order to identify modulation of MnSod expression with aging, we monitored the levels of MnSod transcripts in aged oocytes in response to stressing conditions. Results from real-time RT–PCR revealed higher levels of MnSod mRNA in untreated aged oocytes when compared with young oocytes. Nevertheless, upon OS, MnSod mRNAs decreased to a level similar to that in young untreated oocytes (Fig. 8).

Effect of SIRT1 inhibition on *in vitro* maturation

In order to investigate whether SIRT1 may play a role in meiosis progression, GV oocytes were exposed to different Ex527 concentrations in M16 medium and monitored for meiotic resumption after 16 h. As shown in Table III, meiotic progression was inhibited in a dose-dependent manner. A significant reduction in progression to MII stage was observed at 5 μM (control versus 5 μM Ex527, $P = 0.004$), although the greatest inhibition was observed at higher doses (control versus 10 μM Ex527, $P < 0.001$; 5 μM Ex527 versus 20 μM Ex527, $P = 0.012$). Most of the Ex527 oocytes that did not reach the MII stage were blocked at GVBD. As shown in Table III, GVBD oocytes increased from 24.7% at 10 μM to 37.7% at 20 μM Ex527 (10 μM Ex527 versus 20 μM Ex527, $P = 0.024$). The proportions of oocytes that were degenerated or arrested at GV stage were not different among the groups. Ex527

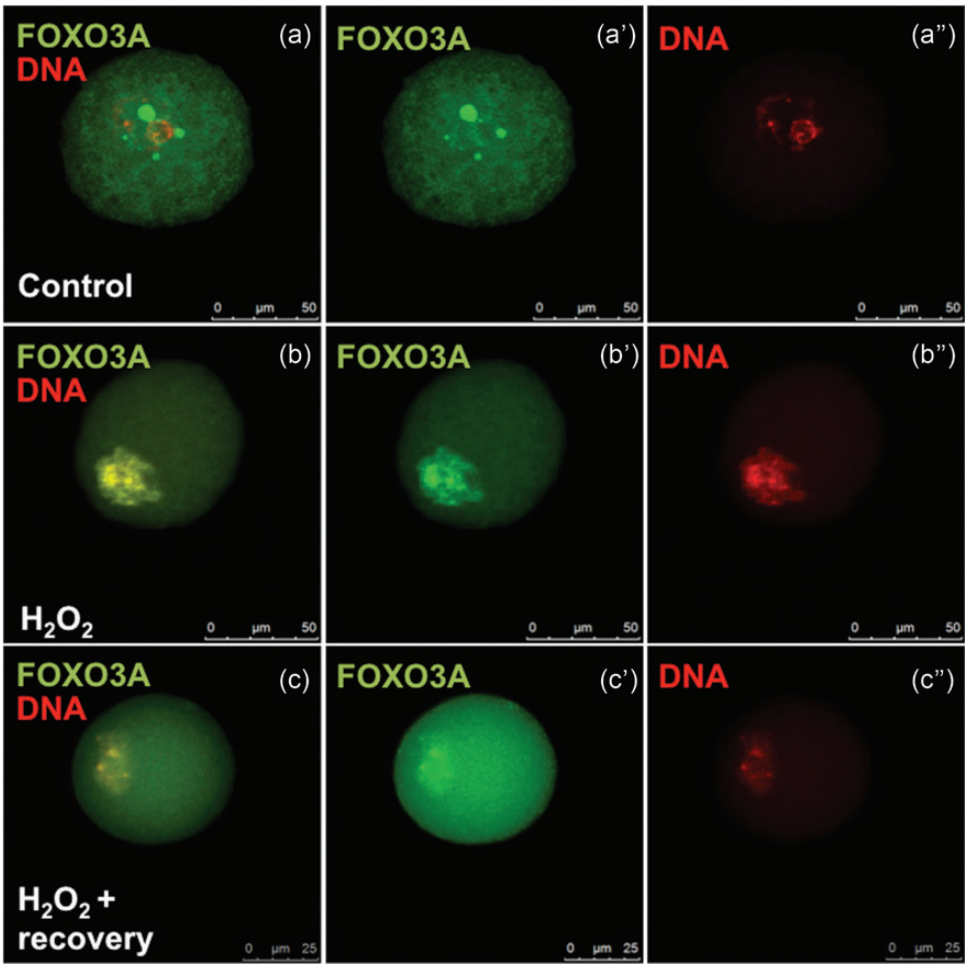

Figure 5 Changes of intracellular localization of FOXO3A after oxidative stress. Micrographs of representative FOXO3A localization in control untreated GV oocytes (**a**–**a**"), in oocytes exposed to H_2O_2 for 30 min during GV-stage arrest by cilostamide (**b**–**b**") and oocytes exposed H_2O_2 for 30 min and cultured to recover for 30 min (**c**–**c**"). These staining patterns were presented in >80% of oocytes analysed in each group.

concentrations higher than 20 µM were not tested to avoid toxic effects of DMSO in which Ex527 was dissolved.

Since normal metaphase configuration is crucial to oocyte developmental competence, we analysed the effect of SIRT1 inhibition on spindle and chromosome organization in *in vitro* matured oocytes. The MII plate was classified as 'normal' when microtubules formed two opposite poles in association with a normal chromosomal distribution or 'aberrant' if microtubule structures displayed reduced dimensions of the spindle or lost normal poles or if disorganized microtubule patterns were observed in association with scattered, decondensed or disorganized chromosomes (Liu and Keefe, 2002). According to this classification, our data showed that the presence of Ex527 during *in vitro* maturation was associated with an increase to almost 25% of oocytes having aberrant MII plates (Supplementary data, Fig. S1).

Since *in vitro* maturation has been associated with an increase in ROS (Combelles *et al.*, 2009), we investigated the role of SIRT1 on the maintenance of the redox state during IVM culture. The analysis was performed only in oocytes that reached the stage of MII. As shown in Table IV, oocytes matured in the presence of 500 µM NAC, a concentration previously used in Kawamura *et al.* (2010), exhibited reduced levels of ROS when compared with control oocytes. By contrast, in cells matured in the presence of the SIRT1 inhibitor, an increase of ROS production was observed. This effect was not observed when medium containing Ex527 was supplemented with NAC (Table IV).

Discussion

Oxidative stress is known to continuously threaten the acquisition and maintenance of oocyte developmental potential during *in vivo* processes and *in vitro* manipulations (Agarwal *et al.*, 2008; Combelles *et al.*, 2009; Tatone *et al.*, 2010). Previous studies in somatic cells have provided strong evidence for the role of SIRT1 as a sensor of cell redox state and a protector against OS and aging (Hori *et al.*, 2013; Hwang *et al.*, 2013; Salminen *et al.*, 2013). Data obtained in the present investigation demonstrate that SIRT1 plays a pivotal role in the adaptive response of mouse GV oocytes to OS and strongly suggest that SIRT1 signalling acts in the front line defence against ROS through the FoxO3a-MnSod axis.

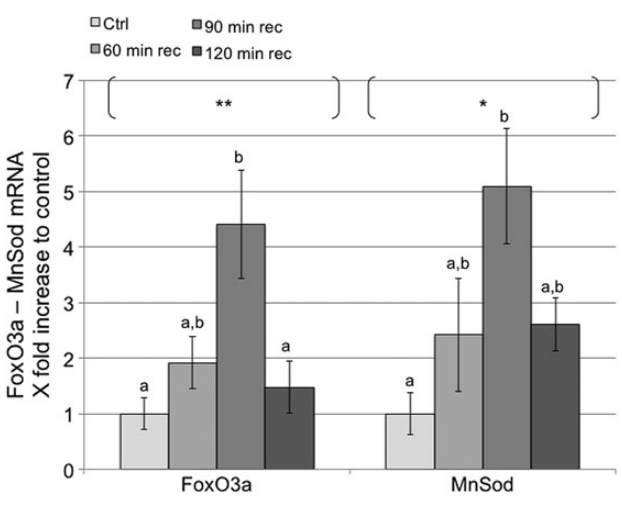

Figure 6 Changes in gene expression of Sirt1-related pathway in oocytes stressed by H_2O_2. Analysis of FoxO3a and MnSod, expression in oocytes exposed to H_2O_2 and cultured to recover for 60, 90 or 120 min. Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.05$, **:$P < 0.01$, one-way ANOVA; a, b: $P < 0.05$, Student Newman Keuls test.

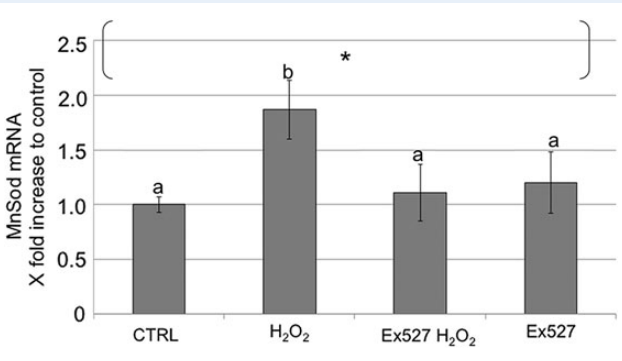

Figure 7 Effect of SIRT1 inhibition on MnSod expression. Ex527-treated oocytes were exposed to H_2O_2 and processed for real-time RT–PCR after 90 min in Ex527 supplemented medium (Ex527-H_2O_2). A group of oocytes were maintained in M16 for 1 h before exposure to H_2O_2 and processed for real-time RT–PCR after 90 min in plain medium (H_2O_2). Another group of oocytes were processed for real-time RT–PCR after 1 h in Ex527 supplemented medium (Ex527). Control oocytes were processed soon after retrieval (CTRL). Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.01$, one-way ANOVA; a, b: $P < 0.05$, Student Newman Keuls test.

Consistent with previous observations by Manosalva and Gonzáles (2010), in our study SIRT1 exhibited a prevalent nuclear localization in mouse GV oocytes. Additionally, we have shown that OS caused

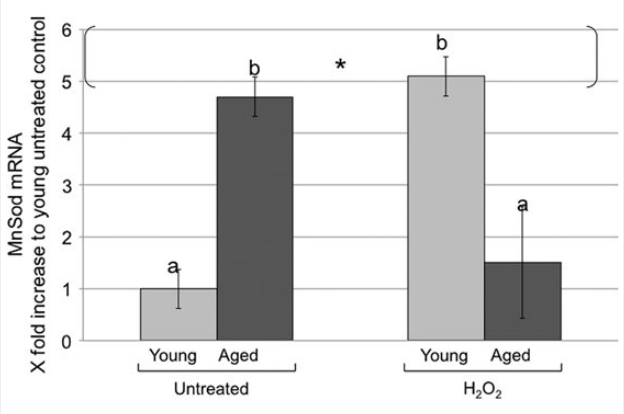

Figure 8 Changes in the expression of MnSod during aging. Analysis of MnSod expression in young and aged oocytes exposed to H_2O_2 for 30 min and then processed after 90 min. Values are means ± SEM of three determinations. The fold change of each gene is shown as $2^{-\Delta\Delta Ct}$. Statistical comparisons were performed by one-way ANOVA and Student Newman Keuls Multiple Comparison, *:$P < 0.01$, one-way ANOVA; a, b: $P < 0.05$, Student Newman Keuls test.

SIRT1 rearrangements inside the cell leading to the appearance of intense cytoplasmic staining along with increased fluorescence in the nucleus. Although the intracellular localization of this enzyme depends on the cell type, nucleocytoplasmic shuttling is considered relevant to its activity (Tanno *et al.*, 2007; Yang *et al.*, 2007; Tong *et al.*, 2013). Thus, our observation of OS effects on SIRT1 localization points to SIRT1 involvement in the OS response and suggests that both nuclear and cytoplasmic targets may participate in SIRT1 signalling (Aquilano *et al.*, 2013). In addition to changes in intracellular localization of SIRT1, we observed some effects of OS on GV chromatin configuration. In the control untreated young oocytes, the percentages of showing NSN (not surrounded nucleolus) and SN (surrounded nucleolus) chromatin were in accordance with the literature (De La Fuente, 2006; Manosalva and Gonzáles, 2010), whereas in the OS group these values decreased and most of oocytes showed the nonNSN and nonSN (nNSN-nSN) chromatin configuration firstly reported by Manosalva and Gonzáles (2010) as a peculiar pattern of GV oocytes from old mice (Supplementary data, Table SII). This observation along with the finding that percentage of nNSN-nSN oocytes in the OS group is higher than in the aged group would support the hypothesis that OS may have a relevant role in the chromatin changes leading to the nNSN-nSN configuration. In addition we can speculate that these modifications may reflect changes in transcriptional activity triggered by OS and related to chromatin regulating factors, including SIRT1.

A body of evidence was found in the present research to support the pivotal role of SIRT1 as a protector from OS in mouse oocytes. Notably, the Sirt1 gene is up-regulated during the OS response reaching peak transcript levels at 90 min post OS treatment followed by a return to basal levels at 180 min. This kinetics is supposed to ensure optimal levels of SIRT1 activity during the adaptive response to OS and homeostasis maintenance. Consistent with this is the observation that this finely tuned mechanism is disrupted in the aged oocyte where lower levels of SIRT1 protein and a lower ability to regulate the Sirt1 gene were detected. As a matter of fact, in contrast to young oocytes, aged

Table III Degeneration, GV-stage meiotic arrest or progression to GVBD or MII stage for oocytes cultured for 16 h in the presence of increasing concentrations of Ex527 ranging from 2.5 to 20 μM.

	Degeneration		Meiotic arrest		Meiotic resumption		
	n	Degeneration (mean % ± SEM)	*n*	GV (mean % ± SEM)	*n*	GVBD (mean % ± SEM)	MII (mean % ± SEM)
Control	124	4 (2.7% ± 3.1)	120	6 (4.4% ± 3.7)	114	9 (6.2% ± 6.8)^{*,a,§}	105 (86.7% ± 12.0)^{*,a,§}
2.5 μM Ex527	73	5 (6.7% ± 11.5)	68	4 (5.2% ± 4.9)	64	8 (10.4% ± 9.8)^{*,a}	56 (77.8% ± 6.0)^{*,a}
5 μM Ex527	66	8 (12.1% ± 6.6)	58	6 (8.9% ± 3.8)	52	13 (19.5% ± 4.0)^{*,ab}	39 (59.6% ± 8.3)^{*,b}
10 μM Ex527	72	12 (16.9% ± 11.2)	60	6 (8.0% ± 10.6)	54	18 (24.7% ± 6.9)^{*,b}	36 (50.3% ± 7.8)^{*,b,c}
20 μM Ex527	155	15 (9.2% ± 9.0)	140	25 (16.1% ± 4.5)	115	58 (37.7% ± 4.4)^{*,c,§}	57 (37.0% ± 6.3)^{*,c,§}

Values are mean percentages ± SEM of at least three independent experiments. Statistical differences among the groups by one-way ANOVA: $^{*}P < 0.001$; multiple pairwise comparison by Student Newman Keuls test: $^{a, b, c}P < 0.05$, $^{§}P < 0.001$.

Table IV Effect of Sirt1 inhibition on *in vitro* maturation.

	n	ROS level‡ (± SEM)
Control oocytes	58	38 778 (± 11 522)^{*,a,§}
20 μM Ex527	61	48 008 (± 11 775)^{*,b}
20 μM Ex527 + 500 μM NAC	64	37 434 (± 9434)^{*,a}
500 μM NAC	51	32 555 (± 11 133)^{*,a,§}

‡Mean pixel intensity of H_2DCF fluorescence in MII oocytes obtained after *in vitro* maturation in the presence of 20 μM Ex527, 20 μM Ex527 + 500 μM NAC, 500 μM NAC or in plain medium (control). Values are means ± SEM of three independent experiments. Statistical differences among the groups by one-way ANOVA: $^{*}P < 0.001$; multiple pairwise comparison by Student Newman Keuls test: $^{a, b}P < 0.001$, $^{§}P < 0.05$.

oocytes exhibited increased levels of Sirt1 mRNAs that could reflect the physiological condition of OS related to aging, as it occurs in in young oocytes exposed to OS. There are a lot of data in the literature pointing to a relevant role of OS in spindle aberrations and susceptibility to meiotic errors, which represent the hallmarks of the reduced competence of aged oocytes (Eichenlaub-Ritter, 2012). Many lines of evidence support this view. First of all, a strong relationship between ROS/advanced glycation end-products (AGEs) in the environment during oocyte growth and maturation and impaired perifollicular or stromal vascularization is known to play a relevant role in the OS status of aged oocytes (Yeh *et al.*, 2008; Tatone *et al.*, 2014). Secondly mouse and human aged oocytes showed altered expression of genes involved in mitochondrial function, oxidative stress, energy pathways, transcription control and stress responses (Hamatani *et al.*, 2004; Steuerwald *et al.*, 2007). On this basis, altered Sirt1 levels and aberrant SIRT1 signalling during the stress response are in line with the current information about genetic and environmental factors underlying oocyte aging.

Based on the above observations, it is not surprising that aged oocytes have a decreased ability to react to H_2O_2 exposure by up-regulating the Sirt1 gene. Nevertheless based on our results, we cannot rule out that aged oocytes would respond to OS more slowly than young oocytes and eventually present late up-regulation of Sirt1 gene.

The age-related disruption of the normal antioxidant response was confirmed by our observation that, in spite of high levels of Sirt1 mRNA, the SIRT1 protein was undetectable in aged oocytes. A shorter half-life of the SIRT1 protein or altered translation mechanisms with aging may account for this condition (Jung *et al.*, 2009).

In recent literature, advanced genomic analysis has revealed an enormous list of functional non-coding RNAs, which are biologically relevant in gene regulation, both at transcriptional and post-transcriptional levels. Among non-coding RNAs, microRNAs (miRNAs) are known to be involved in the regulation of hundreds of target genes, and changes in the expression of even a single miRNA can impact the outcome of multiple cellular activities. Many papers have described the regulatory role of several miRNAs in SIRT1 post-transcriptional regulation (Yamakuchi, 2012), as well as in OS response (Chen *et al.*, 2013). miRNAs are expressed in mouse oocytes and embryos and their deregulation negatively affects regulatory pathways involved in follicle growth and oocyte maturation (Hossain *et al.*, 2012; Assou *et al.*, 2013). In this context, our study represents the first evidence for OS responsive miRNAs in germ cells. We have shown that in the mouse oocyte, either OS or aging induces a decrease in miR-132 expression in concordance with an increase in Sirt1 mRNA. The indirect correlation between the expression levels of these genes along with the observation that Sirt1 is a validated target of miR-132 strongly support the hypothesis that decreased levels of miR-132 contribute to the increase in Sirt1 mRNA during the oocyte OS adaptive response. Nevertheless, the observation that in aged oocytes exposed to OS, Sirt1 and miR-132 levels remained unchanged revealed that this mechanism is severely compromised with aging. Taken together, all these data suggest that the aged oocyte being unable to face stressing insults may abandon detoxification and, perhaps, initiate new signalling pathways, such as the apoptotic one.

According to the literature, one of the mechanisms through which SIRT1 promotes cell survival under OS is by deacetylation of FOXO3A, a transcription factor that activates the expression of MnSod (Caito *et al.*, 2010). Analysis of changes in the intracellular distribution of FOXO3A in mouse oocytes in the current study provided strong evidence for FOXO3A nucleocytoplasmic shuttling upon H_2O_2 exposure. This revealed initial massive recruitment of this protein in the nucleus followed by a return to homogeneous nucleocytoplasmic distribution. Moreover, in accordance with a role of FOXO3A in the antioxidant response, FoxO3a and MnSod mRNAs increased with the same kinetics as Sirt1 transcripts, suggesting a functional relationship among these genes. Importantly, the finding that the increase in MnSod

mRNA was prevented by SIRT1 inhibition by Ex527 strongly suggests that the FoxO3A-MnSod axis acts downstream to SIRT1 activity. Although enzymatic activity was not assessed in our system, this conclusion is supported by data reporting that Ex527 is a specific inhibitor of the SIRT1 catalytic domain, at least two orders of magnitude more potent than other molecules, i.e. sirtinol (Solomon *et al.*, 2006). In addition Ex527 inhibits SIRT1 activity in somatic cells in a dose-dependent manner, and, in contrast to other inhibitors, at the concentrations we used in this study it does not induce cytotoxicity and does not affect enzymatic activity of other sirtuins (Peck *et al.*, 2010).

The key role of MnSod gene was further confirmed by the finding that disrupted SIRT1 signalling in aged oocytes is associated with altered regulation of MnSod mRNA levels. These transcripts, whose levels were more abundant in aged than in young oocytes, dramatically decreased upon OS so confirming the failed adaptive response in aged cells.

Focusing on the effects of SIRT1 inhibition on *in vitro* maturation, we report here that Ex527 negatively affected meiotic progression. In our system, addition of NAC contributes to reduce ROS generated during IVM. The finding that this effect is compromised when SIRT1 is inhibited indicates that SIRT1 exerts a key role against variations of redox states during IVM. Indeed it is well known that fine modulation of the redox state during IVM is crucial to nuclear maturation and normal metaphase configuration (Combelles *et al.*, 2009). In particular, pro-oxidant conditions are known to induce spindle aberrations and increase the percentage of oocytes with chromosome scattering and clumping on the MII plate (Tarín *et al.*, 1996). On the other hand, studies on numerous animal models have shown that proper antioxidant supplementation can improve *in vitro* nuclear maturation and oocyte competence (Choi *et al.*, 2013; Jo *et al.*, 2014; Mukherjee *et al.*, 2014; Wang *et al.*, 2014). Accordingly, here we demonstrated that inhibition of SIRT1 activity caused a significant increase in anomalies of spindle and chromosomal organization in *in vitro* matured oocytes. Overall, our results indicate that SIRT1 activity protects the oocytes during meiotic progression by supporting antioxidant defences, so preventing damage from unscavanged free radicals.

It can be hypothesized that SIRT1 is the sirtuin mainly involved during oogenesis based on the observation that it is expressed at low levels in oocytes that have reached the MII stage, in contrast to SIRT3 whose role in fertilization and early embryo development has recently been demonstrated (Kawamura *et al.*, 2010). Indeed we observed that the levels of Sirt1 transcripts in GV oocytes are higher when compared with MII oocytes (Supplementary data, Fig. S2).

In summary, we demonstrated that SIRT1 is involved in the adaptive response to OS in the mouse oocyte. This pathway relies on the activities of FoxO3a, MnSod and miR-132 and is deregulated with aging. Although hydrogen peroxide is the pro-oxidant stimulus most commonly used in SIRT1 studies, we cannot rule out that H_2O_2 treatments or stressors different from those used in the present study may differently modulate SIRT1 signalling (Kwon and Ott, 2008).

A limitation of our study is that we investigated stress-related intracellular pathways in denuded oocytes so excluding the possible influence of cumulus cells and other follicular compartments. In this regard, the present results should be interpreted with caution and rather considered a background for further investigation on oocyte SIRT1 signalling.

Overall from our study, it emerges that SIRT1 exerts a protective role during *in vitro* meiosis resumption and completion and highlights the need for studying the impact of SIRT1 activators in human IVM. Moreover, our findings provide the rationale for SIRT1 as a key and specific therapeutic target and confirm previous hypothesis by Liu *et al.* (2012) suggesting that SIRT1 pharmacological activation may be a strategy to ameliorate/preserve oocyte competence in oxidative stress-mediated ovarian dysfunctions including aging.

Supplementary data

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

Authors' roles

C.T., A.M.D. and F.A. took part in the study design, supervision and coordination of the study and drafting of the manuscript. G.D., S.F. and M.Vi. took part in the study execution, data collection and discussions. C.D. and M.Ve. took part in the study design, supervision, data analysis of miR-132 regulation, and drafting of the manuscript.

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Conflict of interest

None declared.

References

Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. *Antioxid Redox Signal* 2008;**10**:1375–1403.

Aquilano K, Baldelli S, Pagliei B, Ciriolo MR. Extranuclear localization of SIRT1 and PGC-1α: an insight into possible roles in diseases associated with mitochondrial dysfunction. *Curr Mol Med* 2013;**13**:140–154.

Assou S, Al-edani T, Haouzi D, Philippe N, Lecellier CH, Piquemal D, Commes T, Aït-Ahmed O, Dechaud H, Hamamah S. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. *Hum Reprod* 2013;**28**:3038–3049.

Barbagallo D, Piro S, Condorelli AG, Mascali LG, Urbano F, Parrinello N, Monello A, Statello L, Ragusa M, Rabuazzo AM *et al.* miR-296–3p, miR-298–5p and their downstream networks are causally involved in the higher resistance of mammalian pancreatic α cells to cytokine-induced apoptosis as compared to β cells. *BMC Genomics* 2013;**14**:62.

Bogliolo L, Murrone O, Di Emidio G, Piccinini M, Ariu F, Ledda S, Tatone C. Raman spectroscopy-based approach to detect aging-related oxidative damage in the mouse oocyte. *J Assist Reprod Genet* 2013;**30**:877–882.

Caito S, Rajendrasozhan S, Cook S, Chung S, Yao H, Friedman AE, Brookes PS, Rahman I. SIRT1 is a redox-sensitive deacetylase that is post-translationally modified by oxidants and carbonyl stress. *FASEB J* 2010;**24**:3145–3159.

Chen Z, Shentu TP, Wen L, Johnson DA, Shyy JY. Regulation of SIRT1 by oxidative stress-responsive miRNAs and a systematic approach to identify its role in the endothelium. *Antioxid Redox Signal* 2013;**19**:1522–1538.

Choi WJ, Banerjee J, Falcone T, Bena J, Agarwal A, Sharma RK. Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure. *Fertil Steril* 2007;**88**:1220–1231.

Choi JY, Kang JT, Park SJ, Kim SJ, Moon JH, Saadeldin IM, Jang G, Lee BC. Effect of 7,8-dihydroxyflavone as an antioxidant on *in vitro* maturation of oocytes

and development of parthenogenetic embryos in pigs. *J Reprod Dev* 2013; **59**:450–456.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 2004; **305**:390–392.

Combelles CM, Gupta S, Agarwal A. Could oxidative stress influence the *in-vitro* maturation of oocytes? *Reprod Biomed Online* 2009;**18**:864–880.

Coussens M, Maresh JG, Yanagimachi R, Maeda G, Allsopp R. Sirt1 deficiency attenuates spermatogenesis and germ cell function. *PLoS One* 2008;**3**:e1571.

De La Fuente R. Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Dev Biol* 2006;**292**:1–12.

Eichenlaub-Ritter U. Oocyte ageing and its cellular basis. *Int J Dev Biol* 2012; **56**:841–852.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet* 2004;**13**:2263–2278.

Hennet ML, Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. *Int J Dev Biol* 2012;**56**:819–831.

Hori YS, Kuno A, Hosoda R, Horio Y. Regulation of FOXOs and p53 by SIRT1 modulators under oxidative stress. *PLoS One* 2013;**8**:e73875.

Hossain MM, Salilew-Wondim D, Schellander K, Tesfaye D. The role of microRNAs in mammalian oocytes and embryos. *Anim Reprod Sci* 2012; **134**:36–44.

Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD^+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010;**31**:194–223.

Hwang JW, Yao H, Caito S, Sundar IK, Rahman I. Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radic Biol Med* 2013; **61C**:95–110.

Jo JW, Jee BC, Suh CS, Kim SH. Addition of lysophosphatidic acid to mouse oocyte maturation media can enhance fertilization and developmental competence. *Hum Reprod* 2014;**29**:234–241.

Jung T, Catalgol B, Grune T. The proteasomal system. *Mol Aspects Med* 2009;**30**:191–296.

Kawamura Y, Uchijima Y, Horike N, Tonami K, Nishiyama K, Amano T, Asano T, Kurihara Y, Kurihara H. Sirt3 protects *in vitro*-fertilized mouse preimplantation embryos against oxidative stress-induced p53-mediated developmental arrest. *J Clin Invest* 2010;**120**:2817–2828.

Kwon HS, Ott M. The ups and downs of SIRT1. *Trends Biochem Sci* 2008; **33**:517–525.

Li Q, Geng X, Zheng W, Tang J, Xu B, Shi Q. Current understanding of ovarian aging. *Sci China Life Sci* 2012;**55**:659–669.

Liu L, Keefe DL. Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. *Hum Reprod* 2002;**17**:2678–2685.

Liu J, Liu M, Ye X, Liu K, Huang J, Wang L, Ji G, Liu N, Tang X, Baltz JM *et al.* Delay in oocyte aging in mice by the antioxidant N-acetyl-L-cysteine (NAC). *Hum Reprod* 2012;**27**:1411–1420.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) *Method. Methods* 2001;**25**:402–408.

Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 2001;**107**:137–148.

Manosalva I, González A. Aging changes the chromatin configuration and histone methylation of mouse oocytes at germinal vesicle stage. *Theriogenology* 2010;**74**:1539–1547.

McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR, Lansdorp PM, Lemieux M. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. *Mol Cell Biol* 2003;**23**:38–54.

Meldrum DR. Aging gonads, glands, and gametes: immutable or partially reversible changes? *Fertil Steril* 2013;**99**:1–4.

Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. *Biochem J* 2007;**404**:1–13.

Morris BJ. Seven sirtuins for seven deadly diseases of aging. *Free Radic Biol Med* 2013;**56**:133–171.

Mukherjee A, Malik H, Saha AP, Dubey A, Singhal DK, Boateng S, Saugandhika S, Kumar S, De S, Guha SK *et al.* Resveratrol treatment during goat oocytes maturation enhances developmental competence of parthenogenetic and hand-made cloned blastocysts by modulating intracellular glutathione level and embryonic gene expression. *J Assist Reprod Genet* 2014;**31**:229–239.

Peck B, Chen CY, Ho KK, Di Fruscia P, Myatt SS, Coombes RC, Fuchter MJ, Hsiao CD, Lam EW. SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2. *Mol Cancer Ther* 2010;**9**: 844–855.

Revollo JR, Li X. The ways and means that fine tune Sirt1 activity. *Trends Biochem Sci* 2013;**38**:160–167.

Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci USA* 2004;**101**:15998–16003.

Salminen A, Kaarniranta K, Kauppinen A. Crosstalk between Oxidative Stress and SIRT1: Impact on the Aging Process. *Int J Mol Sci* 2013; **14**:3834–3859.

Sánchez F, Smitz J. Molecular control of oogenesis. *Biochim Biophys Acta* 2012; **1822**:1896–1912.

Schuster S, Penke M, Gorski T, Petzold-Quinque S, Damm G, Gebhardt R, Kiess W, Garten A. Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes. *PLoS One* 2014;**9**:e91045.

Schwer B, Verdin E. Conserved metabolic regulatory functions of sirtuins. *Cell Metab* 2008;**7**:104–112.

Sobinoff AP, Sutherland JM, Mclaughlin EA. Intracellular signalling during female gametogenesis. *Mol Hum Reprod* 2013;**19**:265–278.

Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS, Huber LJ. Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* 2006;**26**:28–38.

Steuerwald NM, Bermúdez MG, Wells D, Munné S, Cohen J. Maternal age-related differential global expression profiles observed in human oocytes. *Reprod Biomed Online* 2007;**14**:700–708.

Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A, Waters KM. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Mol Endocrinol* 2009;**23**:1876–1884.

Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol Reprod Dev* 2003;**66**:143–152.

Takahashi T, Igarashi H, Kawagoe J, Amita M, Hara S, Kurachi H. Poor embryo development in mouse oocytes aged *in vitro* is associated with impaired calcium homeostasis. *Biol Reprod* 2009;**80**:493–502.

Tanner KG, Landry J, Sternglanz R, Denu JM. Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc Natl Acad Sci USA* 2000; **97**:14178–14182.

Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. *J Biol Chem* 2007;**282**:6823–6832.

Tarín JJ, Vendrell FJ, Ten J, Blanes R, van Blerkom J, Cano A. The oxidizing agent tertiary butyl hydroperoxide induces disturbances in spindle organization, c-meiosis, and aneuploidy in mouse oocytes. *Mol Hum Reprod* 1996;**2**:895–901.

Tatone C, Amicarelli F. The aging ovary—the poor granulosa cells. *Fertil Steril* 2013;**99**:12–17.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 2008;**14**:131–142.

Tatone C, Di Emidio G, Vento M, Ciriminna R, Artini PG. Cryopreservation and oxidative stress in reproductive cells. *Gynecol Endocrinol* 2010;**26**:563–567.

Tatone C, Heizenrieder T, Di Emidio G, Treffon P, Amicarelli F, Seidel T, Eichenlaub-Ritter U. Evidence that carbonyl stress by methylglyoxal exposure induces DNA damage and spindle aberrations, affects mitochondrial integrity in mammalian oocytes and contributes to oocyte ageing. *Hum Reprod* 2011;**26**:1843–1859.

Tatone C, Eichenlaub-Ritter U, Amicarelli F. Dicarbonyl stress and glyoxalases in ovarian function. *Biochem Soc Trans* 2014;**42**:433–438.

te Velde ER, Pearson PL. The variability of female reproductive ageing. *Hum Reprod Update* 2002;**8**:141–154.

Tong C, Morrison A, Mattison S, Qian S, Bryniarski M, Rankin B, Wang J, Thomas DP, Li J. Impaired SIRT1 nucleocytoplasmic shuttling in the senescent heart during ischemic stress. *FASEB J* 2013;**27**:4332–4342.

Vogt E, Kipp A, Eichenlaub-Ritter U. Aurora kinase B, epigenetic state of centromeric heterochromatin and chiasma resolution in oocytes. *Reprod Biomed Online* 2009;**19**:352–368.

Wang F, Tian X, Zhang L, He C, Ji P, Li Y, Tan D, Liu G. Beneficial effect of resveratrol on bovine oocyte maturation and subsequent embryonic development after *in vitro* fertilization. *Fertil Steril* 2014;**101**:577–586.

Wiersma A, Hirsch B, Tsafriri A, Hanssen RG, Van de Kant M, Kloosterboer HJ, Conti M, Hsueh AJ. Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. *J Clin Invest* 1998;**102**:532–537.

Wood JW. Fecundity and natural fertility in humans. *Oxf Rev Reprod Biol* 1989;**11**:61–109.

Yamakuchi M. MicroRNA Regulation of SIRT1. *Front Physiol* 2012;**3**:68.

Yang Y, Fu W, Chen J, Olashaw N, Zhang X, Nicosia SV, Bhalla K, Bai W. SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. *Nat Cell Biol* 2007;**9**:1253–1262. Erratum in: *Nat Cell Biol* 2007;**9**:1442.

Yeh J, Kim BS, Peresie J. Ovarian vascular endothelial growth factor and vascular endothelial growth factor receptor patterns in reproductive aging. *Fertil Steril* 2008;**89**:1546–1556.