Reproductive BioMedicine Online (2014) ■■, ■■–■■

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats

Vijayakumar Govindaraj, KB Rajani, AJ Rao *

Department of Biochemistry, Indian Institute of Science, Bangalore, India
* Corresponding author. *E-mail address:* ajrao@biochem.iisc.ernet.in (AJ Rao).

Professor Rao obtained his PhD in Biochemistry in 1971 from the Indian Institute of Science, Bangalore, with post-doctoral work at the Universities of California and Sherbrooke. His main research area is hormonal regulation of cellular proliferation and differentiation using placental and Leydig cells as models. He has contributed significantly to research on evaluation of contraceptives, and has established the efficacy of the use of 7alpha-methyl-19-nortestosterone and immunization against FSH receptor peptides as an approach to male contraception. Professor Rao has authored over 158 scientific publications, and is currently working as Senior Scientist at the Indian Institute of Science, Bangalore, India.

Abstract Oocytes present at birth undergo a progressive process of apoptosis in humans and other mammals as they age. Accepted opinion is that no fresh oocytes are produced other than those present at the time of birth. Studies have shown that DNA repair genes in oocytes of mice and women decline with age, and lack of these genes show higher DNA breaks and increased oocyte death rates. In contrast to the ethical problems associated with monitoring the changes in DNA double-strand breaks in oocytes from young and old humans, it is relatively easy to carry out such a study using a rodent model. In this study, the mRNA levels of DNA repair genes are compared with protein products of some of the genes in the primordial follicles isolated from immature (18–20 days) and aged (400–450 days) female rats. Results revealed a significant decline in mRNA levels of *BRAC1* ($P < 0.01$), RAD51 ($P < 0.05$), ERCC2 ($P < 0.05$), and H2AX ($P < 0.01$) of DNA repair genes and phospho-protein levels of BRAC1 ($P < 0.01$) and H2AX ($P < 0.05$) in primordial follicles of aged rats. Impaired DNA repair is confirmed as a mechanism of oocyte ageing.

KEYWORDS: *BRCA1*, DNA repair, H2AX, ovarian aging, primordial follicles, RAD51

Introduction

One of the important challenges in women's reproductive health is the age-related decline in reproductive performance (Ottolenghi et al., 2004). The duration of the female fertile life span is influenced by the number of diplotene oocytes, which are surrounded by a single layer of cuboidal granulosa cells arrested in the first meiotic prophase stored in the ovary as primordial follicles (Hirshfield, 1991; Tingen et al., 2009). The rate of decline in primordial follicle reserve increases with age, resulting in cessation of reproduction and menopause (Kato et al., 1998; Kerr et al., 2013). As ovarian reserve and fertility declines with age, pregnancy failure also increases, and meiotic errors lead to chromosomally

http://dx.doi.org/10.1016/j.rbmo.2014.11.010
1472-6483/

abnormal conceptions (Adhikari and Liu, 2009). Most follicles undergo atresia throughout postnatal life, mainly through apoptosis (Kim and Tilly, 2004). It has been shown that internucleosomal DNA fragmentation, which is a hallmark of cells undergoing apoptosis, occurs in atretic follicles (Tilly, 1996), along with the production of DNA double-strand breaks (DSB), triggering the mitochondrial apoptotic pathway (Roos and Kaina, 2006). The integrity of oocyte genome in mammals is affected by two distinctive methods: the meiotic recombination of homologous chromosomes during the fetal life; and a long postnatal period of meiotic arrest (dictyate stage), before completing meiotic divisions (De Felici and Klinger, 2011). Meiotic recombination results in DNA DSB. These are generally repaired by the oocyte at the end of the meiotic prophase I (Ashwood-Smith and Edwards, 1996). During arrested stage of primordial follicle (dictyate stage), the DNA of the oocytes is subjected to a wide range of potential damages, whose effects can be highly detrimental to female fertility (Ashwood-Smith and Edwards, 1996; Hanoux et al., 2007; Mira, 1998). Several in-vivo and in-vitro studies suggest that the mammalian oocytes are capable of repairing DNA damage, namely DSB, produced by meiotic recombination or by external agents (De Felici and Klinger, 2011). Failure to repair DNA damage caused by recombination triggers meiotic checkpoints and disposal of the germ cells by apoptosis (Cohen et al., 2006). The underlying mechanism for age-induced decline of ovarian follicle reserve and related DNA damage in primordial follicles is largely unknown. A recent study by Titus et al. (2013), however, has reported an impaired BRAC1 related ATM-mediated DNA DSB repair as a cause of ageing in mouse and human oocytes and a decline of oocyte repair efficiency with age. In contrast to the ethical problems associated with monitoring the changes in genes and proteins involved in repair of DNA DSB in oocytes from young and old humans, it is relatively easy to carry out such a study using a rodent model. The maximum fertility in the rat occurs between 100–300 days of age, and breeding activity usually begins at 100–120 days of age. Although female rats do not experience the menopause, the absence of regular oestrus cycle (equivalent to the menopause, which occurs around 51–52 years in humans) is observed between 450 and 540 days, but breeding efficiency is reduced well before (i.e. to about 250 days [8–10 months]) (Durbin et al., 1966). Rats older than 1 year have delayed pregnancy, decreased litters, trouble in delivery and more bleeding during delivery; hence, the age of 18–20 days was chosen for selecting immature rats, and 450 days for adult rats that are no longer capable of reproduction. In the present study, the level of expression of genes and protein products of two of the genes involved in the repair of DNA DSB were monitored in young animals (in which the primordial follicles are not subjected to hormone action) and in very old animals (in which the effect of hormones are minimal and are no longer capable of reproduction).

Materials and methods

Animals

Immature (18–20 days) and aged (400–450 days) Wistar strain albino rats were obtained from the Central Animal Facility at the Indian Institute of Science, Bangalore. The experimental procedures carried out using these animals were approved by the Institutional Animal Ethics Committee constituted under the guidelines of CPCSEA, Ministry of Environment and Forests, Government of India, New Delhi (Institutional Animal Ethics Committee Project number: CAF/Ethics/270/2012, approved 13 July 2012) Animals were kept in a 12-h light–dark cycle and provided *ad libitum* with water and a laboratory diet.

Isolation of rat primordial follicles

Primordial follicles were isolated as described by Shi et al. (2007). Briefly, immature (18–20 days) and aged (400–450 days) female Wistar rats were killed by excess CO2, and the ovaries were dissected out, free of adhering fat, into a petri-dish containing cold phosphate-buffered saline (PBS). The ovaries were washed twice in PBS containing penicillin (100 units/ml) and streptomycin (100 μg/ml), and were minced into small pieces, transferred to a digestion medium containing 4 ml of Dulbecco's Modified Eagle's Medium (Sigma, Cat. No.D0422) supplemented with fetal bovine serum at a final concentration of 4% (Biological Industries, Cat. No. 04-127-1A) and 5 ml of PBS and 2% collagenase type IV (Calbiochem, Cat No. 234153). The suspension was incubated at 37°C for 30 min in a shaking water bath, and the follicular digest was filtered through a 70-μm nylon cell strainer (SPL Life Science, Korea) into a 50-ml Falcon tube. The follicular filtrate was centrifuged at 2300 × g for 5 min at 4°C and the supernatant was decanted. The pellet was re-suspended in fresh 5 ml of PBS, and suspension was passed through a 40-μm nylon cell strainer followed by centrifugation at 2300 × g for 5 min at 4°C. The pellet was finally re-suspended in about 400–600-μl Dulbecco's Modified Eagle's Medium (supplemented with 4% fetal calf serum), and the suspension was checked for presence of the primordial follicles under an inverted phase-contrast microscope. The isolated primordial follicles were considered morphologically normal if they appeared with a round oocyte surrounded by one compact layer of granulosa cells as observed under a microscope. For immature rats, the yield of primordial follicles was about 35,000 per rat and a total of 70 rats were used for isolation of RNA and protein for each experiment. In the case of aged rats, a total of 120 rats were used for each experiment, and the yield of primordial follicles was 10,000 per rat.

Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA from the primordial follicles was isolated using TRIzol reagent (Sigma-number T9424) according to the manufacturer's recommended protocol. The quality and quantity of RNA are assessed using ultraviolet spectrophotometric analysis, by monitoring A280–A260 ratio. The quality of isolated RNA was also assessed by 1% formaldehyde agarose gel electrophoresis using 1% MOPS as gel running buffer. Total RNA was reverse transcribed using ReverseAid First Strand cDNA synthesis kit (Thermoscientific, USA, number 1621). Polymerase chain reaction (PCR) was carried out using 2X PCR

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

Table 1 List of primers used.

Gene	*Primers (5'-3')*	*Size (in bp)*	*Annealing Temp (°C)*
BRCA1	FP: CCAGAAAAATGTCCGCGTAT RP: ACTGTCAGCCCATCTGCTCT	**201**	60
BRCA2	FP: TGGGGAGAATGAGATTGAGG RP: TCCAAGAGAGAAACGCCACT	**246**	60
MRE11	FP: CAGAAAGAGGGATGGGTGA RP: GCCTCTCGAACTTCATCGTC	**217**	60
RAD51	FP: TTTGGAGAATTCCGAACTGG RP: GACGCTTGGTAAAGGAGCTG	**248**	60
ATM	FP: GGCACAGAAGAGGGAGACTG RP: TGACGGGAAATATGGTGGAT	**226**	60
ERCC2	FP: GGAATTTGACACCCATGGAC RP: GCCAGCTCTTTGGATACCAG	**201**	60
H2AX	FP: TCCAGAAATCTCGGCCCTTT RP: CGGCCTACAAATGACGACAG	**249**	60
GAPDH	FP: CTCATGACCACAGTCCATGC RP: TTCAGCTCTGGGATGACCTT	**155**	60

Master Mix (Thermoscientific, USA, number K0172) with 1 μL of cDNA and 20 μM of specific forward and reverse primers (Sigma Genosys, Sigma Aldrich, India) in 50 μL reaction. The details of primers and the conditions used in real time (PCR) analysis are given in **Table 1**. To confirm PCR amplification, PCR product was mixed with six times DNA gels loading dye and subjected to electrophoresis on 1% agarose gel containing 1% ethidium bromide (5 μl/100 ml) at constant voltage (80 V) for 60 min in 0.5X trisi-borate ethylene-diamine-tetraacetic acid buffer. The amplified PCR product was visualized as a single compact band of expected size under ultraviolet light and documented by Alpha Digi Doc Gel Documentation and Image Analysis System (Alpha Innotech Corporation, San Leandro, CA). Densitometric analysis of the real-time PCR from three independent experiments after *GAPDH* normalization is shown in the bar graph (mean ± SEM).

Western blotting

Freshly isolated primordial follicles from immature and adult rats were re-suspended in radioimmunoprecipitation assay buffer buffer (50 mM Tris,150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100 and protease inhibitor cocktail) (Roche Diagnostics). Homogenization was conducted using a hand-held teflon-glass homogenizer. The homogenate was centrifuged at 2800 × g at 4°C for 10 min and the protein in supernatant was estimated spectrophotometrically by Lowry's method (Lowry et al., 1951). From each group, 40 μg of protein was electrophorsed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blot analysis was carried out as described earlier (Rama et al., 2001). The blots were incubated over night with primary antibodies (rabbit polyclonal anti-phospho-BRCA1 (Ser1524) from Cell Signaling Technologies (Cat. number 9009; dilution 1:1000; 220 kDa), rabbit polyclonal anti-phospho-histone H2A.X (Ser139) (Cell Signaling Technologies Cat. No. 9718; dilution 1:500;14 kDa) and anti-actin C-2 (Santa Cruz Biotechnology, Cat. No. sc-8432; dilution 1:1000; 42 kDa). Secondary antibody (i.e. goat anti-rabbit) (Cat. number NA934, GE Healthcare) or anti-mouse (Cat. number NA931, GE Healthcare) at dilution 1:5000 were used. The blots were visualized using enhanced chemiluminescence (ECL prime, GE Healthcare) according to the manufacturers' instructions. The chemiluminescence was detected using X-ray films. Exposure time of membranes was adjusted until clearly visible signal could be seen. Densitometric analysis of the western blot from three independent experiments after normalization with actin is presented in the bar graph (mean ± SEM).

Statistics

Statistical analysis of arbitrary units derived from densitometric measurements of DNA and protein bands from the study was carried out using SPSS software (Version 13.0; SPSS Inc, Chicago, USA). All the data are presented as the mean ± SEM ($n = 3$). Differences between groups were calculated by Student's t-test and $P < 0.05$ was considered as statistically significant.

Results

Down-regulation of expression of key DNA DSB repair genes in adult rat primordial follicle

The level of expression of DNA DSB repair genes *BRAC1* (Breast Cancer 1, early onset), *BRCA2* (Breast Cancer 2, early onset), RAD51 (RAD radiation repair gene), MRE11 (meiotic recombination 11 homolog A), ATM (ataxia telangiectasia mutated), ERCC2 (excision repair cross-complementing rodent repair deficiency, complementation group 2) and H2AX (histone H2A) in immature and aged rat primordial follicles assessed by semi-quantitative real time PCR are presented in **Figure 1**. As can be seen, the expression of mRNA for *BRCA1* is significantly decreased (**$P < 0.01$ versus immature), RAD51 (*$P < 0.05$ versus

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

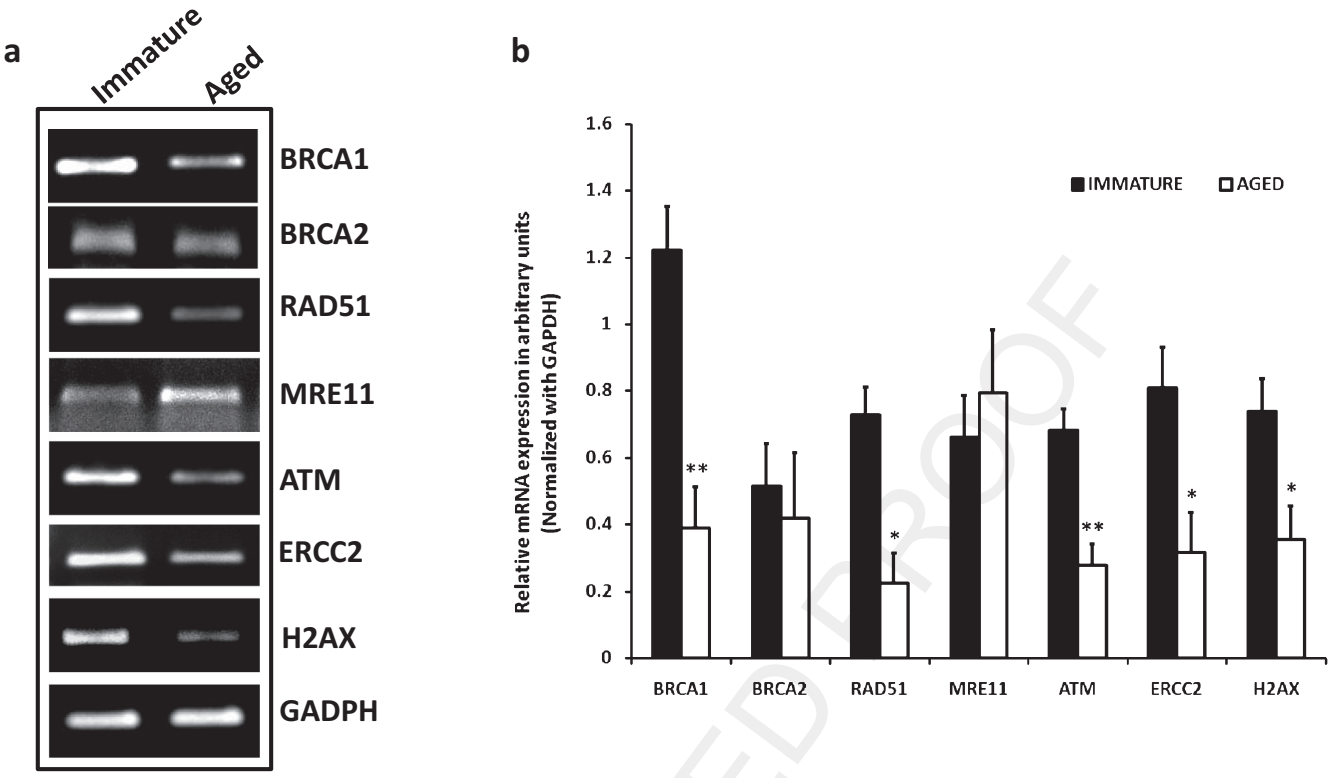

Figure 1 Semi-quantitative real-time polymerase chain reaction analysis of DNA double-strand break repair genes. Total RNA isolated from immature and aged rat primordial follicles, reverse transcribed, and the complementary DNA was subjected to semi-quantitative real-time polymerase chain reaction in a linear range of amplification with GAPDH as an internal control. Representative image of three independent experiments are shown in Panel A. A graphical representation of results is presented in panel B. Data from three independent experiments were expressed as arbitrary densitometric units (mean ± SEM). *$P < 0.05$, **$P < 0.01$ compared with immature rat primordial follicles.

immature), ATM ($P < 0.01$ versus immature), ERCC2 ($P < 0.05$ versus immature) and H2AX ($P < 0.05$ versus immature) in primordial follicles from aged rats compared with immature rats. More than a two-fold decrease was observed in the expression of *BRCA1* and RAD 51 and marginal but significant decrease was seen in the case of ERCC2 and H2AX, although no significant change in the expression of mRNA levels of *BRCA2* and MRE11 between immature and aged primordial follicles was observed.

Decreased levels of phosphorylation of BRCA1 (ser 1524) and H2AX in adult rat primordial follicles

Western blot analysis for phosphorylated BRCA1 and H2AX in immature and adult primordial follicles was carried out using antibodies recognizing Ser-1524 of BRAC1 (~220 kDa) and Ser-139 of γH2AX (~15 kDa). It is evident from the results presented in **Figure 2** that a significant decrease has occurred in the expression levels of phospho-BRCA1 (*$P < 0.01$ versus immature) and phospho-H2AX (*$P < 0.05$ versus immature) in primordial follicles isolated from aged rats compared with immature rats.

Discussion

It is well established that, although women are born with over a million oocytes, only 400 to 500 mature depending on when the woman reaches menopause (Wallace and Kelsey, 2010). Currently, it is accepted that, from birth to menopause, no fresh oocytes are produced other than those that are present at birth (Gleicher et al., 2011; Wallace and Kelsey, 2010). It is known that chromosomal abnormalities increase in the oocytes with age (Handyside et al., 2012; Jessberger, 2012; Jones and Lane, 2012; Nagaoka et al., 2012). A recent study revealed that, in humans and rodents, DSB increase in primordial follicles with age and a corresponding decrease in expression of the critical DNA DSB repair genes occur, namely *BRAC1* and MRE-11, RAD51 and ATM (Titus et al., 2013). The results of the present study using a rodent model confirm and extend those findings. It may be pertinent to point out that the conclusions of Titus et al. (2013), although important and interesting, are based on quantitative PCR and immunohistochemistry results. We have been able to provide results from both real-time PCR as well as western blot studies, which essentially confirms their results and supports their conclusions. Furthermore, it was also reported that, in *BRCA1* deficient mice, reproductive capacity was impaired, the count for primordial follicles was lower and interestingly an increase in DSB in the remaining follicles was found to increase with age (Oktay et al., 2010; Rzepka-Gorska et al., 2006; Valentini et al., 2013). On the basis of these results, it was suggested that DNA DSB repair efficiency is an important determinant of oocyte aging in women. As mentioned earlier, considering the fact that such an analysis in young and old women is ethically not acceptable, the expression of

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

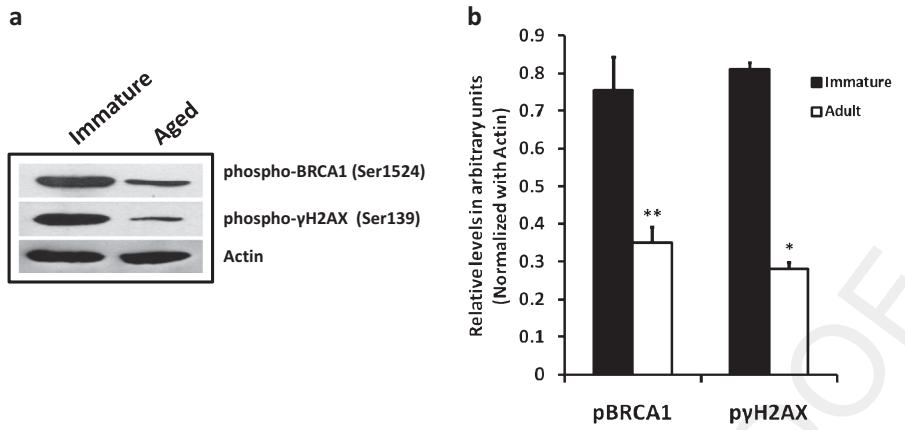

Figure 2 Western blot analysis for phosphorylated BRCA1 (Ser-1524) and H2AX (Ser 139). A total of 40 μg of protein from each sample separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotted using monoclonal antibodies to phospho-BRCA1 (220 kDa) and phospho-H2AX (15 kDa), then immunoreactivity visualized by enhanced chemiluminescence (Panel A). A graphical representation of results is presented in panel B. Data from three independent experiments were expressed as arbitrary densitometric units (mean ± SEM). *$P < 0.05$, **$P < 0.01$ compared with immature rat primordial follicles.

mRNA levels of DNA DSB repair genes was analysed, as well as some of the phosphorylated forms of the two important enzymes by western blot in primordial follicles isolated from immature and aged rats. The present results reveal a significant decrease in the expression of DNA repair genes, namely *BRAC1*, RAD-51, ATM, ERCC2, and H2AX in primordial follicles isolated from aged rats. It is known that phophorylation of BRCA1 directly controls DNA repair and its loss leads to genome instability (Coene et al., 2005; Gatei et al., 2000; Ouchi, 2006; Summers et al., 2011). As assessed by western blot analysis, our results demonstrated a significant decrease in the phosphorylated form of BRCA1 (ser 1524), which indicates a decline in BRCA1-related DNA repair mechanism in primordial follicles from aged rats compared with immature rats. Titus et al. (2013) used whole mice ovarian sections to immunolocalize γH2AX and BRCA1. They reported an increase in gammaH2AX and decrease in BRCA1; however, it should be emphasized that the evidence from H2AX null mutant, which showed inefficient DNA repair in the absence of H2AX and its associated foci (Celeste et al., 2002), strongly suggest both the BRCA1 and phosphorylated form of H2AX are necessary for repair of DSB. The decline in phosphorylated form of H2AX at Ser139, which is necessary for repair of DNA-DSB, as well as decline in the BRCA1 as assessed by real-time PCR and western blot in the present study, suggests that the efficiency of DNA repair machinery is impaired in aged rat primordial follicles. It is also demonstrated that H2AX significantly overlaps with BRCA1 after DNA damage, and BRCA1-γ-H2AX foci are thought to be sites of DNA repair (Paull et al., 2000). Immunolocalization of phospho-proteins was not carried out in the current study owing to financial constraints.

Similar to BRCA1, H2AX, which is also a tumour suppressor helping to preserve genome integrity and is rapidly phosphorylated on serine 139 (denoted as γH2AX), showed a significant decrease in primordial follicles isolated from aged rats. The decrease in phosphorylated form of both BRCA1 and H2AX shows a lack of recruitment of proteins necessary during repair of DSB in primordial follicles isolated from aged rats.

Age-related changes are normally prominent in the expression of genes involved in DNA damage response and repair apart from control of cell cycle and energy pathways (Eichenlaub-Ritter, 2012). It has been shown that human oocytes express DNA repair genes at elevated levels allowing low tolerance for DNA decays (Menezo et al., 2007, 2010). Studies by Rzepka-Gorska et al. (2006) and others (Oktay et al., 2010; Valentini et al., 2013) have demonstrated the role of *BRCA1* in ovarian ageing as seen by diminished DNA repair, a decline in ovarian reserve and interestingly women who carry *BRCA1* mutations undergo menopause prematurely. Altered ovarian function is seen in BRCA mutation carriers, along with reduced response to ovarian hyper-stimulation. Oktay et al. (2010) demonstrated a reduced number of oocytes in patients diagnosed with breast cancer and undergoing fertility preservation. They were able to demonstrate this in patients with the BRCA1 mutation but not with mutation in BRCA2. The investigators postulated that, because DNA repair is defective in BRCA1 mutations, impairment in the oocyte DNA repair is the probable cause of the reduced number of oocytes when these patients are subjected to ovarian stimulation (Oktay et al., 2010). BRCA1 is a substrate for kinases such as ATM, ATR (ataxia telangiectasia and Rad3 related) and Chk1 (checkpoint kinase-1) in response to DNA damage. For BRCA1 phosphorylation, ATM is important, and both $BRCA1^{-/-}$ and $ATM^{-/-}$ cells share many similarities in phenotype, including abnormalities in G2-M-phase checkpoint control and sensitivity to treatment with DNA damaging agents (Xu et al., 1999). Slow growth and premature ageing occurs in ATM-deficient cells, and patients with ataxia telangiectasia do not show hyperphosphorylation of BRCA1 after gamma-irradiation induced DNA damage, suggesting that phosphorylation depends on ATM activity (Hande et al., 2001). In the present study, the decline in ATM and phospho-BRCA1 (Ser 1524) indicate a defect in DNA repair efficiency in aged primordial follicles compared with immature primordial follicles. In addition, ATM also phosphorylates histone H2AX (γH2AX), which forms a platform for the recruitment of necessary checkpoint and DNA repair proteins (Celeste et al., 2002; Paull et al., 2000) and γH2AX is usually robust during apoptosis (Solier and Pommier, 2009). In the absence of H2AX and its associated foci, H2AX null mutant showed inefficient DNA repair (Celeste et al.,

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

2002). This strongly suggests that H2AX is necessary during repair of DSB. It has also been shown that H2AX significantly overlaps with *BRCA1* after DNA damage, and *BRCA1*-γ-H2AX foci are thought to be sites of DNA repair (Paull et al., 2000).

Both BRCA1 and BRCA2 regulate repair of DSB and maintain genomic integrity in response to DNA damage and co-localize with RAD51, a protein which assist in DNA repair (Yoshida and Miki, 2004). In the present study, the expression of *BRCA2* was not altered in aged primordial follicles. Experimental evidence shows that BRCA2 is a key RAD51 interacting partner, BRCA2-RAD51 complex was thought to have two possible mechanisms in dealing with DNA repair, either the prevention of RAD51 binding to single strand DNA during repair or transferred to the site of DNA damage by BRCA2. The observed decrease in RAD51 without change in BRCA2 requires more detailed study of BRCA2-RAD51 complex. The studies on RAD51, however, clearly show its role in homologous recombination and DNA repair. Embryonic lethality are shown in RAD51 knockout mice, and deletion of RAD51 in various cell lines results in extreme sensitivity to DNA-damaging agents, defective DSB repair and, ultimately, cell death (Sonoda et al., 1998; Tsuzuki et al., 1996). Also ageing affects the expression of RAD51 significantly in bovine oocytes (Bilotto et al., 2013). RAD51 interacts also with BRCA1, and plays a major role in homologous recombination of DNA during double strand break repair and act as an important determinant of oocyte ageing in women (Kim et al., 2012; Smirnova et al., 2004; Titus et al., 2013). Similar to RAD51 recombinase activity, MRE11 nuclease activity is also considered as an important DSB repair enzyme. These form a complex consisting of MRE11, Rad50, and Nbs1 (Nibrin 1), and function in diverse aspects of the cellular response to DNA damage (Nickerson et al., 1990). In the present study, no difference was seen in MRE11 levels of immature and adult rat primordial follicles. In contrast to this, another important DNA repair enzyme RAD51 was significantly reduced in aged primordial follicles. This decline in RAD51 also supports results obtained by Kujjo et al. (2010), which demonstrated that RAD51 is one of the candidates shown to have critical role in the repair of DNA damage in oocytes of both chemotherapy-induced and spontaneously induced DNA damage by the ageing process.

In addition to analysing the expression of BRCA1, RAD51, MRE-11 and ATM genes, an attempt was made to investigate the expression of nucleotide excision repair gene ERCC2 (excision repair cross-complementing group 2 also called xeroderma pigmentosum group D-XPD). Our real-time PCR analysis showed ERCC2 expression was significantly lower in primordial follicles from aged rats compared with immature rats. The protein encoded by ERCC2(XPD) is an integral member of basal transcription factor BTF2/TFIIH complex, which is an essential element in the repair of both damaged bases and single strand breaks (Drapkin and Reinberg, 1994; Iyer et al., 1996; Weber et al., 1990). ERCC2/XPD has been shown to have ATP-dependent DNA helicase activity, and is involved in nucleotide excision repair of DNA by opening DNA around the damage (Clarkson and Wood, 2005; Schaeffer et al., 1994). It is pertinent to note in this connection that mice with a mutation in XPD exhibit premature ageing (De Boer et al., 2002).

Our results, which reveal a decline in message and protein levels of BRCA1 and other DNA repair genes in aged rat primordial follicles, allow us to postulate that rat immature primordial follicles possess a robust machinery of DNA repair to DSB. These findings clearly support the evidence of impaired DNA repair as a mechanism of oocyte ageing, and DNA repair is very important for maintenance of oocyte quality. Although documented evidence is not available on the effect of in-vitro ageing of oocytes on the DNA repair mechanisms in assisted reproduction procedures, some studies have reported using rodent and bovine oocytes (Koyama et al., 2014; Lacham-Kaplan and Trounson, 2008; Li et al., 2014). The results of the studies with rodent and bovine oocytes indicate the developmental potential of in-vitro matured oocytes is decreased with increasing chromosomal aberration and incorrect spindle assembly. Considering this, it should be noted that female fertility critically depends on the ability of oocytes to repair the damaged DNA, which is important for the preservation of the oocyte pool.

Acknowledgements

The author A.J. Rao would like to thank the Indian National Science Academy for the award of INSA Senior Scientist Fellowship. The author also wishes to thank the Department of Biochemistry, Indian Institute of Science, Bangalore, India. Department of Biotechnology, Government of India (Project No. BT/PR5487/AAQ/1/500/2012) for financial assistance provided during the course of the work.

References

Adhikari, D., Liu, K., 2009. Molecular mechanisms underlying the activation of mammalian primordial follicles. Endocr. Rev. 30, 438–464.

Ashwood-Smith, M.J., Edwards, R.G., 1996. DNA repair by oocytes. Mol. Hum. Reprod. 2, 46–51.

Bilotto, S., Boni, R., Russo, G., Lioi, M., 2013. Meiosis progression and donor age affect expression profile of DNA repair genes in bovine oocytes. Zygote 1–8.

Celeste, A., Petersen, S., Romanienko, P.J., Fernandez-Capetillo, O., Chen, H.T., Sedelnikova, O.A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M.J., Redon, C., Pilch, D.R., Olaru, A., Eckhaus, M., Camerini-Otero, R.D., Tessarollo, L., Livak, F., Manova, K., Bonner, W.M., Nussenzweig, M.C., Nussenzweig, A., 2002. Genomic instability in mice lacking histone H2AX. Science 296, 922–927.

Clarkson, S.G., Wood, R.D., 2005. Polymorphisms in the human XPD (ERCC2) gene, DNA repair capacity and cancer susceptibility: an appraisal. DNA Repair (Amst) 4, 1068–1074.

Coene, E.D., Hollinshead, M.S., Waeytens, A.A., Schelfhout, V.R., Eechaute, W.P., Shaw, M.K., Van Oostveldt, P.M., Vaux, D.J., 2005. Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. Mol. Biol. Cell 16, 997–1010.

Cohen, P.E., Pollack, S.E., Pollard, J.W., 2006. Genetic analysis of chromosome pairing, recombination, and cell cycle control during first meiotic prophase in mammals. Endocr. Rev. 27, 398–426.

De Boer, J., Andressoo, J.O., De Wit, J., Huijmans, J., Beems, R.B., Van Steeg, H., Weeda, G., Van Der Horst, G.T., Van Leeuwen, W., Themmen, A.P., 2002. Premature aging in mice deficient in DNA repair and transcription. Science 296, 1276–1279.

De Felici, M., Klinger, F.G., 2011. DNA damage and apoptosis in fetal and ovarian reserve oocytes. In: Cell Death in Mammalian Ovary. Springer.

Drapkin, R., Reinberg, D., 1994. The multifunctional TFIIH complex and transcriptional control. Trends Biochem. Sci. 19, 504–508.

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

Durbin, P.W., Williams, M.H., Jeung, N., Arnold, J.S., Parrott, M.W., Davis, T., 1966. Development of spontaneous mammary tumors over the life-span of the female Charles River (Sprague-Dawley) rat: the influence of ovariectomy, thyroidectomy, and adrenalectomy-ovariectomy. Cancer Res. 26, 400–411.

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

Gatei, M., Scott, S.P., Filippovitch, I., Soronika, N., Lavin, M.F., Weber, B., Khanna, K.K., 2000. Role for ATM in DNA damage-induced phosphorylation of BRCA1. Cancer Res. 60, 3299–3304.

Gleicher, N., Weghofer, A., Barad, D.H., 2011. Defining ovarian reserve to better understand ovarian aging. Reprod. Biol. Endocrinol. 9, 23.

Hande, M.P., Balajee, A.S., Tchirkov, A., Wynshaw-Boris, A., Lansdorp, P.M., 2001. Extra-chromosomal telomeric DNA in cells from Atm(-/-) mice and patients with ataxia-telangiectasia. Hum. Mol. Genet. 10, 519–528.

Handyside, A.H., Montag, M., Magli, M.C., Repping, S., Harper, J., Schmutzler, A., Vesela, K., Gianaroli, L., Geraedts, J., 2012. Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation. Eur. J. Hum. Genet. 20, 742–747.

Hanoux, V., Pairault, C., Bakalska, M., Habert, R., Livera, G., 2007. Caspase-2 involvement during ionizing radiation-induced oocyte death in the mouse ovary. Cell Death Differ. 14, 671–681.

Hirshfield, A.N., 1991. Development of follicles in the mammalian ovary. Int. Rev. Cytol. 124, 43–101.

Iyer, N., Reagan, M.S., Wu, K.-J., Canagarajah, B., Friedberg, E.C., 1996. Interactions involving the human RNA polymerase II transcription/nucleotide excision repair complex TFIIH, the nucleotide excision repair protein XPG, and Cockayne syndrome group B (CSB) protein. Biochemistry 35, 2157–2167.

Jessberger, R., 2012. Age-related aneuploidy through cohesion exhaustion. EMBO Rep. 13, 539–546.

Jones, K.T., Lane, S.I., 2012. Chromosomal, metabolic, environmental, and hormonal origins of aneuploidy in mammalian oocytes. Exp. Cell Res. 318, 1394–1399.

Kato, I., Toniolo, P., Akhmedkhanov, A., Koenig, K.L., Shore, R., Zeleniuch-Jacquotte, A., 1998. Prospective study of factors influencing the onset of natural menopause. J. Clin. Epidemiol. 51, 1271–1276.

Kerr, J.B., Myers, M., Anderson, R.A., 2013. The dynamics of the primordial follicle reserve. Reproduction 146, R205–R215.

Kim, M.R., Tilly, J.L., 2004. Current concepts in Bcl-2 family member regulation of female germ cell development and survival. Biochim. Biophys. Acta 1644, 205–210.

Kim, T.M., Ko, J.H., Hu, L., Kim, S.A., Bishop, A.J., Vijg, J., Montagna, C., Hasty, P., 2012. RAD51 mutants cause replication defects and chromosomal instability. Mol. Cell. Biol. 32, 3663–3680.

Koyama, K., Sung-Sik, K., Huang, W., Yanagawa, Y., Takahashi, Y., Nagano, M., 2014. Aging-related changes in in vitro-matured bovine oocytes: oxidative stress, mitochondrial activity and ATP content after nuclear maturation. J. Reprod. Dev. 60, 136.

Kujjo, L.L., Laine, T., Pereira, R.J., Kagawa, W., Kurumizaka, H., Yokoyama, S., Perez, G.I., 2010. Enhancing survival of mouse oocytes following chemotherapy or aging by targeting Bax and Rad51. PLoS ONE 5, e9204.

Lacham-Kaplan, O., Trounson, A., 2008. Reduced developmental competence of immature, in-vitro matured and postovulatory aged mouse oocytes following IVF and ICSI. Reprod. Biol. Endocrinol. 6, 58.

Li, M., Zhao, H.C., Li, R., Yu, Y., Qiao, J., 2014. Chromosomal aberrations in in-vitro matured oocytes influence implantation and ongoing pregnancy rates in a mouse model undergoing intracytoplasmic sperm injection. PLoS ONE 9, e103347.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Menezo, Y., Dale, B., Cohen, M., 2010. DNA damage and repair in human oocytes and embryos: a review. Zygote 18, 357–365.

Menezo, Y., Jr., Russo, G., Tosti, E., El Mouatassim, S., Benkhalifa, M., 2007. Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. J. Assist. Reprod. Genet. 24, 513–520.

Mira, A., 1998. Why is meiosis arrested? J. Theor. Biol. 194, 275–287.

Nagaoka, S.I., Hassold, T.J., Hunt, P.A., 2012. Human aneuploidy: mechanisms and new insights into an age-old problem. Nat. Rev. Genet. 13, 493–504.

Nickerson, J.A., Krockmalnic, G., He, D., Penman, S., 1990. Immunolocalization in three dimensions: immunogold staining of cytoskeletal and nuclear matrix proteins in resinless electron microscopy sections. PNAS 87, 2259–2263.

Oktay, K., Kim, J.Y., Barad, D., Babayev, S.N., 2010. Association of BRCA1 mutations with occult primary ovarian insufficiency: a possible explanation for the link between infertility and breast/ovarian cancer risks. J. Clin. Oncol. 28, 240–244.

Ottolenghi, C., Uda, M., Hamatani, T., Crisponi, L., Garcia, J.E., Ko, M., Pilia, G., Sforza, C., Schlessinger, D., Forabosco, A., 2004. Aging of oocyte, ovary, and human reproduction. Ann. N. Y. Acad. Sci. 1034, 117–131.

Ouchi, T., 2006. BRCA1 phosphorylation: biological consequences. Cancer Biol. Ther. 5, 470–475.

Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M., Bonner, W.M., 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr. Biol. 10, 886–895.

Rama, S., Suresh, Y., Rao, A., 2001. Regulation of telomerase during human placental differentiation: a role for TGFβ1. Mol. Cell. Endocrinol. 182, 233–248.

Roos, W.P., Kaina, B., 2006. DNA damage-induced cell death by apoptosis. Trends Mol. Med. 12, 440–450.

Rzepka-Gorska, I., Tarnowski, B., Chudecka-Glaz, A., Gorski, B., Zielinska, D., Toloczko-Grabarek, A., 2006. Premature menopause in patients with BRCA1 gene mutation. Breast Cancer Res. Treat. 100, 59–63.

Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J., Egly, J., 1994. The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. EMBO J. 13, 2388.

Shi, K., Yuan, X., Du, L., Pan, D., Zhang, Y., Zhao, Y., Deng, X., Hu, X., Wu, C., Li, N., 2007. Advanced methods of isolation and identification of porcine primordial follicles. Anim. Reprod. Sci. 101, 163–171.

Smirnova, M., Van Komen, S., Sung, P., Klein, H.L., 2004. Effects of tumor-associated mutations on Rad54 functions. J. Biol. Chem. 279, 24081–24088.

Solier, S., Pommier, Y., 2009. The apoptotic ring. Cell Cycle 8, 1853–1859.

Sonoda, E., Sasaki, M.S., Buerstedde, J.M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., Takeda, S., 1998. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J. 17, 598–608.

Summers, K.C., Shen, F., Sierra Potchanant, E.A., Phipps, E.A., Hickey, R.J., Malkas, L.H., 2011. Phosphorylation: the molecular switch of double-strand break repair. Int. J. Proteomics 2011, 373816.

Tilly, J.L., 1996. Apoptosis and ovarian function. Rev. Reprod. 1, 162–172.

Tingen, C., Kim, A., Woodruff, T.K., 2009. The primordial pool of follicles and nest breakdown in mammalian ovaries. Mol. Hum. Reprod. 15, 795–803.

Titus, S., Li, F., Stobezki, R., Akula, K., Unsal, E., Jeong, K., Dickler, M., Robson, M., Moy, F., Goswami, S., Oktay, K., 2013. Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. Sci. Transl. Med. 5, 172ra21.

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010

Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., Morita, T., 1996. Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. Proc. Natl. Acad. Sci. U.S.A. 93, 6236–6240.

Valentini, A., Finch, A., Lubiński, J., Byrski, T., Ghadirian, P., Kim-Sing, C., Lynch, H.T., Ainsworth, P.J., Neuhausen, S.L., Greenblatt, E., 2013. Chemotherapy-Induced Amenorrhea in Patients With Breast Cancer With a BRCA1 or BRCA2 Mutation. J. Clin. Oncol. 31, 3914–3919.

Wallace, W.H., Kelsey, T.W., 2010. Human ovarian reserve from conception to the menopause. PLoS ONE 5, e8772.

Weber, C.A., Salazar, E.P., Stewart, S., Thompson, L., 1990. ERCC2: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast RAD3. EMBO J. 9, 1437.

Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T., Deng, C.X., 1999. Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol. Cell 3, 389–395.

Yoshida, K., Miki, Y., 2004. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. Cancer Sci. 95, 866–871.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 21 July 2014; refereed 14 November 2014; accepted 18 November 2014.

Please cite this article in press as: Vijayakumar Govindaraj, K.B. Rajani, A.J. Rao, Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats, Reproductive BioMedicine Online (2014), doi: 10.1016/j.rbmo.2014.11.010