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MINI-REVIEW ***Developments in reproductive biology and medicine***

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Novel insights into reproductive ageing and menopause from genomics

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ABSTRACT: The post-reproductive phase or menopause in females is triggered by a physiological timer that depends on a threshold of follicle number in the ovary. Curiously, reproductive senescence appears to be decoupled from chronological age and is instead thought to be a function of physiological ageing. Ovarian ageing is associated with a decrease in oocyte developmental competence, attributed to a concomitant increase in meiotic errors. Although many biological hallmarks of general ageing are well characterized, the precise mechanisms underlying the programmed ageing of the female reproductive system remain elusive. In particular, the molecular pathways linking the external menopause trigger to the internal oocyte chromosome segregation machinery that controls fertility outcomes is unclear. However, recent large scale genomics studies have begun to provide insights into this process. Next-generation sequencing integrated with systems biology offers the advantage of sampling large datasets to uncover molecular pathways associated with a phenotype such as ageing. In this mini-review, we discuss findings from these studies that are crucial for advancing female reproductive senescence research. Targets identified in these studies can inform future animal models for menopause. We present three potential hypotheses for how external pathways governing ovarian ageing can influence meiotic chromosome segregation, with evidence from both animal models and molecular targets revealed from genomics studies. Although still in incipient stages, we discuss the potential of genomics studies combined with epigenetic age acceleration models for providing a predictive toolkit of biomarkers controlling menopause onset in women. We also speculate on future research directions to investigate extending female reproductive lifespan, such as comparative genomics in model systems that lack menopause. Novel genomics insights from such organisms are predicted to provide clues to preserving female fertility.

Key words: genomics / menopause / reproductive ageing / ovarian reserve / epigenetic age clock

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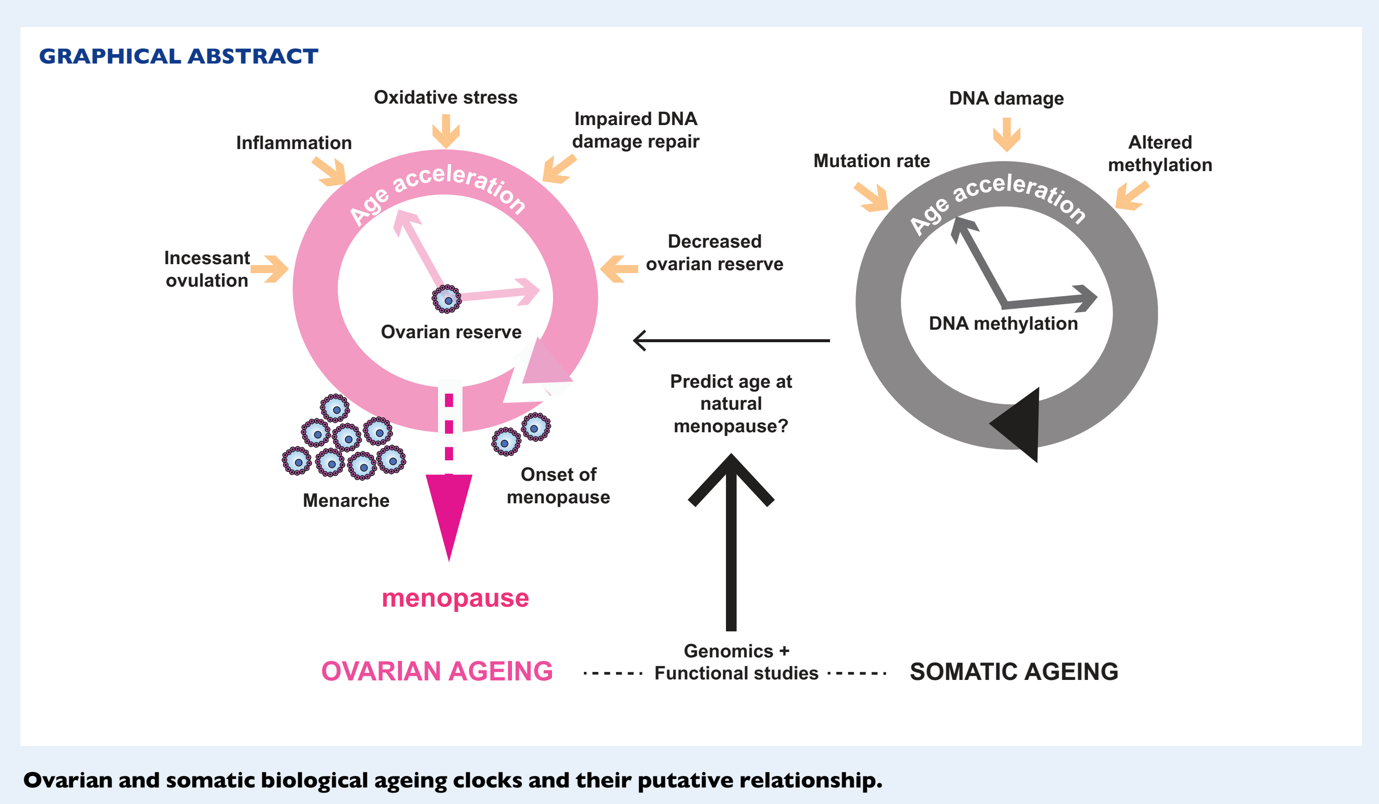
GRAPHICAL ABSTRACT



Ovarian and somatic biological ageing clocks and their putative relationship.

Introduction

Universally, ageing is a progressive decline of physiological integrity resulting in functional deterioration. Many biological processes are established hallmarks of ageing across mammalian species including genomic instability, epigenetic changes, telomere shortening, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopez-Otin *et al.*, 2013). Female reproductive ageing is unique in terms of its apparent disconnection with the onset of ageing processes in the other organs. The most intriguing aspect is that the ovary operates a physiological timer or clock which depends on the number of non-growing follicles that comprise the ovarian reserve after birth, enforcing reproductive senescence when a threshold of 1000 follicles remain in the ovary (te Velde and Pearson, 2002; Broekmans *et al.*, 2009; Wallace and Kelsey, 2010). Numerous outstanding questions exist regarding what determines the follicular threshold that controls the female reproductive lifespan. Undeniably, female reproductive ageing research will benefit from defining the biological hallmarks specific to the female reproductive system, which remain elusive to date. Developments in genomics are beginning to elucidate the cell type and developmental stage-specific molecular players that constitute the biological clock of female fertility. However, our understanding of how it works to time the reproductive life span, and how its molecular components interact with environmental factors to irreversibly shut down the reproductive system before other systems, is far from complete. Understanding this clock and its constituents will spark the development of strategies to mitigate the impact of female reproductive ageing on women's reproductive choices. In this mini-review, we not only address new findings from genomics but also discuss the importance of building a strong bridge with comparative and molecular biology to accelerate discovery and development of predictive or anti-ageing strategies.

Genomics identifies pathways that operate the ovarian biological clock: insights into the irreversible end of a woman's reproductive life

The relationship between natural fertility and age follows an inverse U-shaped curve with reduced rates at both age extremes (Hawkes and Smith, 2010). Chromosome segregation errors during meiosis were shown to follow the same U-shaped trend, with increased frequencies in teenage and advanced maternal age (AMA) women (Gruhn *et al.*, 2019) and were proposed to be the main determinant of female fecundity. Based on these observations, the hypothesis of a molecular meiotic chromosome driven 'timing' mechanism of ovarian ageing, such as cohesion weakening (discussed below), was brought forward (Gruhn *et al.*, 2019). The critical role of oocyte quality as a determinant in reproductive ageing is best demonstrated in IVF cycles where women of AMA who use oocytes from reproductively young donors increase their birth rate per embryo transferred from 4% in autologous transfers to ∼25% (HFEA report, 2020). But is oocyte genome integrity the only driver of fecundity decline as females age? The very

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elegant hemi-ovariectomy surgical experiments in rodents (discussed in the next section) show that the size of the initial oocyte pool determines the time of onset of both ovarian senescence and oocyte aneuploidy (Brook *et al.*, 1984; Nelson and Felicio, 1986).

Which mechanisms associate with variation of the age at menopause onset in humans? Analytical dissection of the genetic associations from genome-wide association studies (GWAS) of age at natural menopause (ANM) (Day *et al.*, 2015; Ruth *et al.*, 2021) yielded novel insights into these themes. Genetics contribute to >50% of the variation in the ANM onset (Snieder *et al.*, 1998; Murabito *et al.*, 2005) and significantly associated common variants explain up to 32.4% of the UK biobank genotype array heritability and 10% of ANM variance in an independent cohort (Ruth *et al.*, 2021). Further elegant integrative analyses including an expression quantitative trait locus (QTL) survey for 48 tissues, network analysis, pathway enrichment and gene expression quantitation, fine mapped the GWA loci to genes mediating molecular pathways including DNA damage response (DDR), apoptosis and metabolic signaling (Ruth *et al.*, 2021). Integrative genomics and systems concepts such as gene network inference from single-cell expression studies in foetal primordial germ cells (PGCs), oocytes and granulosa cells (GCs) from primordial, primary, secondary antral follicle and mature metaphase II (MII) oocytes yielded novel insights into the temporal activation of each mechanism from foetal to adult life (Ruth *et al.*, 2021). Importantly, we now have more accurate information on the varied insults which occur in different developmental stages, follicular events and cell types (oocytes versus GCs), and which invoke specific DDR mechanisms (Day *et al.*, 2015; Ruth and Murray, 2016; Ruth *et al.*, 2021). For example, genetic variation in genes mediating single- and double-strand break (DSB) repair, mismatch repair (*MSH5*, *MSH6*), base excision repair, DNA damage checkpoint kinase genes (*CHEK1*, *CHEK2*) and repair of replication stress-induced inter-strand crosslinks (*BRCA1*, *FANCA*, *FANCB*, *FANCM*, *FAAP24*) is expected to impact the development of foetal oocytes and follicular growth at different stages (Ruth *et al.*, 2021). Similarly, variation in apoptotic genes (*TP63*, *BCL2*, *BCL2L11*, *PPARG*, *PPP5C*) located within 300 kb of the single-nucleotide polymorphisms with the strongest statistical association for ANM, suggests varying activation downstream of DDR, probably regulating attrition and depletion of the ovarian reserve.

Can the size of the follicular endowment be maintained at optimal levels at birth and can we delay depletion of the ovarian reserve over time? Experiments in mice show that manipulation of CHEK1 and CHEK2 increases the reproductive lifespan, and women carrying loss-of-function variants in *CHEK2* reported an ANM almost 3.5 years later than non-carriers (Ruth *et al.*, 2021). Through these experiments, we gained deeper knowledge on the activity of specific DDR mechanisms in the establishment and the depletion of the ovarian reserve (discussed in the next section). Several DDR ANM-associated genes (*MSH5*, *MSH6*, *BRCA1*, *BRCA2*, *FANCA*, *FANCB*, *RAD51*) are important for genomic integrity surveillance in the foetal germline where diverse DDR mechanisms engage various pathways to repair different lesions across ovarian development. Meiotic DDR pathways target errors during homologous recombination and play a pivotal role in the surveillance of genome integrity during meiosis I where foetal oocytes appear to escape the 'metaphase I check point' (Lenzi *et al.*, 2005). The size of the follicular reserve is determined at birth by 'clearing' defective oocytes during the perinatal attrition period (Martinez-Marchal *et al.*, 2020). Importantly, pre-meiotic DDR-mediated pathways also clears defective PGCs (Hill and Crossan, 2019). When unrepaired DNA lesions accumulate in PGCs, because of a defective or absent inter-strand cross-link repair pathway, the affected cells are cleared by apoptosis during a very narrow developmental window during epigenetic reprogramming and proliferation. Metabolic insults, such as the impaired catabolism of aldehydes, cause DNA damage to PGCs (Hill and Crossan, 2019). In contrast, the surrounding gonadal somatic cells do not differ in the frequency of DNA DSBs (Hill and Crossan, 2019). This observation indicated that perhaps mutagenic insults in somatic cells are less frequent than in PGCs. Hence there is a decreased requirement for repair in the soma compared to the germline, which is in line with purifying selection preventing the development of germline cells passing mutations to the next generation.

DNA repair has emerged as a universal functional cog of the ageing clock, by quantifying the mutation rate-defined as the number of mutations per year, across 16 mammalian species (Cagan *et al.*, 2022). The somatic mutational rate is inversely correlated with lifespan (Cagan *et al.*, 2022), suggesting that the DNA repair mechanisms are more efficient in longer-lived species, which in turn indicates that a conserved somatic tissue biological clock also relies on DNA repair.

What do we know about DNA damage in the adult ovary? Temporal expression of the genes implicated in the NAD+ salvage pathway (*NAMPT*, *PPARG*, *FOXO1*, *SIRT1*) and in caloric restriction (*IGF1*) through the IGF1-axis across the consecutive follicular developmental stages suggests a link between environmental inducers of age-related DNA damage which then triggers the DDR and apoptosis. SIRT1 sensing of NAD+ is a central mediator of the anti-ageing benefits of caloric restriction. The association between SIRT1 sensing NAD+ and defective DNA repair has been shown in accelerated neurodegeneration in Cockayne syndrome (Scheibye-Knudsen *et al.*, 2014). Caloric restriction preserves female fertility in aged mice by reducing meiotic spindles defects and by maintaining mitochondrial distribution and ATP production in mature MII oocytes (Selesniemi *et al.*, 2011). Importantly, exposures to an obesogenic diet, both *in utero* and after weaning, affects *Dmc1* recombinase and *Brsk1* DNA damage sensor expression (Ruth *et al.*, 2021).

Single-cell RNA-seq changes in non-human primates modelling human reproductive ageing (pre- and peri-menopausal stages) investigated the impact of age (in a cell-type distinctive manner) on the expression of genes that regulate protection from oxidative stress (*GPX1*, *GSR*), preserve mitochondrial function in oocytes from the early follicular stages, and mediate the protective response to oxidative damage and apoptosis in GCs (*IDH1*, *NDUFB10*) (Wang *et al.*, 2020). Furthermore, DNA oxidation, DNA damage and apoptosis in GCs increased in ageing oocytes compared to those from young animals (Wang *et al.*, 2020).

Collectively, these seminal genomics studies provide critical links between the central ageing molecular mechanism, the DDR, in timing the onset of ovarian senescence in animal models. The link predicts that a disturbance in the balance between DNA damage and repair leads to increased follicular attrition rates either *in utero* or during adult life. In the following sections, we address recent and propose future experiments in models of reproductive ageing that aim at further delineating the mechanistic link between the size of ovarian reserve and the pathways highlighted by genomics studies.

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Genomics and chromosome biology of reproductive senescence: how strong is the bridge?

Increased meiotic aneuploidy has also been reported to be a function of reduced ovarian reserve count from both mouse models of menopause and in humans, although other factors confound the data obtained from humans (Brook *et al.*, 1984; Finch, 2014). This suggests that external physiological factors such as ovulation frequency, follicular atresia and follicle count also underpin the quality of ovulated oocytes. Indeed, an intriguing aspect of reproductive senescence is that it is thought to be a function of physiological rather than chronological ageing since mice oocytes age over months whereas human oocytes age over decades. At the level of chromosome segregation, it is well-established that one of the major causes of the maternal age effect is declining levels of chromosome-associated proteins that are not replenished with age (Chiang *et al.*, 2010). This includes but is not limited to cohesins, like Rec8, Shugoshin-like 2 (SGO2), that ensure centromeric cohesion (Liu and Keefe, 2008; Duncan *et al.*, 2012), and certain actin cytoskeleton proteins that support the oocyte spindle (Dunkley and Mogessie, 2022). A recent exome sequencing study found that genes functioning in centriole formation like Cep120, and DNA damage repair were enriched in blastocysts with higher incidence of maternally derived aneuploidy (Tyc *et al.*, 2020; Wartosch *et al.*, 2021). Likewise, the oocyte-specific spindle protein TUBB8, was also found to cause female infertility (Feng *et al.*, 2016). Altogether, the aetiology of human oocyte aneuploidy has been attributed to inherent meiotic spindle instability, increased merotelic attachments, and age-related changes in kinetochore and chromosome architecture (Fellmeth *et al.*, 2015; Zielinska *et al.*, 2015, 2019; Thomas *et al.*, 2021). However, how the external ovarian changes communicate to the internal segregation machinery remains unclear. Identifying and defining the molecular networks that control these ovarian processes will be key to delaying maternal ageing. Genomics studies have revealed several pathways that are implicated in determining the ANM and the next steps for the field will be testing and validating the role of these target pathways functionally *in vivo*. In this section, we will discuss some models based on functional studies that provide a link between external physiological changes in an ageing ovary and fidelity of the oocyte chromosome segregation. Indeed, some of these studies support findings from the GWAS.

The ovulation suppression model

A recent study tested the hypothesis that cohesion dysfunction is linked to ovulation frequency. Ovulation is an intensely inflammatory process that generates reactive oxygen species and leads to oxidative damage (Duffy *et al.*, 2019). Under this hypothesis, physiological ageing associated with repeated ovulation can cause protein turnover leading to increased segregation errors. Using three mouse models that converge on reducing ovulation frequency, Chatzidaki and colleagues (Chatzidaki *et al.*, 2021) showed that ovulation suppression does indeed reduce aneuploidy by preserving centromeric cohesion in aged oocytes. Thus, a direct molecular consequence of repetitive ovulation cycles is that it negatively impacts oocyte segregation and hence reproductive outcomes. Methods that can reduce ovulation frequency are thus predicted to have a protective effect against reproductive senescence, e.g. usage of oral hormonal contraception. The study by Chatzidaki and colleagues, along with a few others in humans, supports oral contraceptives as a potential clinical method to preserve fertility in women (Janerich *et al.*, 1976; Farrow *et al.*, 2002; Chatzidaki *et al.*, 2021). However, the risks associated with long-term hormonal treatments must be weighed against the benefits of extending reproductive lifespan, especially for younger mothers. The hypothesis that inflammation associated with incessant ovulation exacerbates ageing is further supported by the fact that ablation of pro-inflammatory cytokines such as interleukin 1 results in increased fertility in mice at 2.5 months of age that persists until 12 months of age (Uri-Belapolsky *et al.*, 2014), suggesting that inflammation is one of the molecular determinants of the ANM (Foley *et al.*, 2021). Indeed, a link was experimentally established between the accumulating tissue damage from repeated ovulation in nulliparous 12-month-old virgin mice with gradually increasing ovarian fibrosis and ovulation rate decline compared to 12-month-old repeat breeders (Umehara *et al.*, 2022). The same group showed that administration of pirfenidone (a TGFb1 suppressor, anti-oxidant and anti-inflammatory drug) used to treat pulmonary fibrosis resulted in reproductively aged mice being able to ovulate as opposed to the untreated controls. Moreover, the ovulated oocytes were reproductively competent resulting in blastocyst formation following IVF (Umehara *et al.*, 2022).

Although this study outlines the deleterious effects of repeated ovulation on internal chromosome-associated proteins, there are some proteins like the centromeric histone CENP-A, that are impervious to it. CENP-A, is a histone H3 variant that marks the location of the centromere where a kinetochore is built for spindle attachment and segregation. Therefore, preserving and transmitting CENP-A nucleosomes through the female germline to progeny is essential for chromosome inheritance (Das *et al.*, 2017). Remarkably, CENP-A persists in mouse oocyte chromatin throughout their reproductive lifespan and shows little decline with age (Smoak *et al.*, 2016). It is not known how the centromeric nucleosome is retained through reproductive senescence, unlike Rec8 and SGO2. Since the centromeres, designated by CENP-A, are crucial for meiotic divisions and beyond, perhaps the oocytes have evolved mechanisms to preserve this information long term, and protect against loss of centromere identity in progeny. Indeed, any reduction in centromere strength in one generation is completely recovered in the female germline of progeny possibly through increased CENP-A assembly during oogenesis to compensate for age-related loss (Das *et al.*, 2022). Investigating mechanisms that retain long lived oocyte proteins through prophase arrest will also provide insights into what maintains oocyte quality over time (Das *et al.*, 2017).

Reduced ovarian reserve or follicle counter model

The above model cannot explain all age-related oocyte aneuploidy. Therefore, there must be other drivers of the female fecundity decline. It has been proposed that the reduced follicle count with age can have consequences on the remaining oocytes in terms of aneuploidy (Fu *et al.*, 2014), but the evidence for this in humans is conflicting (Kline *et al.*, 2004, 2011; Honorato *et al.*, 2015). Under the follicle counter model, reduced ovarian reserve even in young females would be

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sufficient to yield high rates of oocyte aneuploidy. The hemi-ovariectomy surgical experiments in rodents have shown that the initial oocyte pool determines both ANM and oocyte aneuploidy (Brook *et al.*, 1984; Nelson and Felicio, 1986). Surgical depletion of primordial follicles is associated with progressively earlier times of ovarian senescence and accelerated aneuploidy onset relative to the size of the ovarian reserve. These experiments suggest that factors such as DNA damage (discussed below), ovulation frequency and pathways controlling follicular attrition, which determine the size of the ovarian reserve, can directly influence oocyte chromosome segregation and reproductive outcomes. However, the molecular links between reduced follicle count and chromosome aneuploidy, if any, are still unknown. It may be worthwhile to revisit such mouse models with earlier onset of ovarian senescence in order to ascertain whether chromosome aneuploidy is directly related to a decreased ovarian reserve size, independent of natural ageing.

Women with either diminished ovarian reserve (DOR) or premature ovarian insufficiency (POI) have reduced follicle count in common, despite showing disparate clinical symptoms (Studer *et al.*, 1984; Greene *et al.*, 2014; Practice Committee of the American Society for Reproductive Medicine, 2020). Both conditions are associated with multiple genetic factors that affect the quantity of oocytes, identified through whole-exome sequencing (Jaillard *et al.*, 2020; Tang and Yu, 2020), but whether the quality of the remaining oocytes is affected in terms of preserving segregation fidelity, is unknown. An attractive option to parse out the molecular links between the follicle counter mechanism and oocyte quality is to leverage the myriad genetic determinants of DOR and/or POI identified from GWA studies (Chon *et al.*, 2021). These studies identified SNPs in genes associated with DNA repair (*BRCA1*, *BRCA2*, *CHEK2*), meiosis (*SYCE1, STAG3*) and the different pathways controlling oocyte growth and folliculogenesis (*GDF9*, *FIGLA*; Qin *et al.*, 2015). As of now, the field uses aged mouse models to study the links between reduced ovarian reserve and oocyte chromosome segregation fidelity. Studying aged animals has the disadvantages of having limited material resulting in reduced statistical power, other detrimental effects from chronological ageing that confound physiological ageing defects, expense and labour intensity. Utilizing mouse models of candidate genes known to induce premature ovarian senescence, provides the advantage of assessing the detrimental effects of physiological ageing on oocyte segregation fidelity in an otherwise young female. Such models can decouple physiological ageing defects from chronological ones, do not require long experimental times, and provide valuable insight into molecular pathways governing premature ovarian senescence.

Harnessing the power of genomics in organisms that lack reproductive senescence also represents an exciting future direction for the field that could uncover the molecular components of the follicle counter mechanism. One such organism is the mammal, the naked mole rat, whose social colonies contain both reproductive (queen) and non-reproductive females. The females suppress ovulation until they are designated to become the queen after which they are fertile for their entire lifespan (Buffenstein, 2005). They also carry a large ovarian reserve (Place *et al.*, 2021), which may contribute to their extended fertility, suggesting that multiple pathways involving both ovulation suppression and large follicle reserves may be required to extend their reproductive potential. Future efforts on comparative genomics between reproductive (queen) and non-reproductive females can provide novel genetic targets specific to the queen that are crucial for extending the reproductive lifespan.

Oocyte quality control checkpoint model

The total number of follicles in human females peaks at 20 weeks of gestation, after which the number dramatically reduces at birth to 300 000–400 000 follicles, which are then cyclically recruited upon menarche (Block, 1953). Menopause is initiated when this number declines over time to ∼1000 (te Velde and Pearson, 2002). Recent genomics studies have revealed an essential role for DNA damage repair (DDR) proteins such as BRCA1 and HELB, in regulating the ANM (Day *et al.*, 2015; Ruth *et al.*, 2021). Notably, ANM-associated mutant variants were also found in seven binding partners of BRCA1 suggesting that homologous recombination repair is pivotal for ovarian ageing. However, variants in mismatch repair (MSH5, MSH6) and base excision repair (APEX1 and PARP2) proteins, were also represented in the data set as being linked with ANM (Day *et al.*, 2015). These proteins constitute a canonical surveillance mechanism that detects genome instability and activates the DNA damage checkpoint at the G2/M boundary of the cell cycle (Gould and Nurse, 1989). It is not surprising that oocytes, which are arrested in G2, have co-opted DDR proteins to monitor genome integrity. Multiple lines of evidence support a critical role of the DDR pathway in oocyte quality control and reproductive ageing.

First, DNA damage checkpoint proteins control the process of follicular atresia thus regulating both follicle count and oocyte quality. Indeed, *Chek2* knock-out mice had lower rates of ovarian reserve depletion and responded with higher numbers of oocytes following hormonal stimulation in comparison to the wild-type aged controls (Ruth *et al.*, 2021), consistent with the role of *Chek2* in culling mouse oocytes with damage (Bolcun-Filas *et al.*, 2014; Tuppi *et al.*, 2018). However, *Chek2* knock-out females were fertile with equivalent litter sizes to controls, suggesting that the DNA damage that CHEK2 responds to while inducing atresia does not irreversibly damage the genome. This suggests that oocytes that are normally culled are not necessarily poor quality oocytes. It remains a mystery as to why the DDR checkpoint eliminates such oocytes and what the selection criteria is. On the other hand, *Chek1* overexpression led to an increased ovarian reserve at birth which delayed the time of onset of ovarian senescence in mice (Ruth *et al.*, 2021).

Second, reduced dosage of DDR proteins (e.g. FANCA and BRCA1) is linked to POI, indicating that proteins in this pathway impact physiological ageing. Indeed, *FANCA* hypomorphs and *BRCA1* deficient mice show reduced follicle count and decreased reproductive potential (Lin *et al.*, 2017; Pan *et al.*, 2021). Women carrying *BRCA1* mutations also show an accelerated decline in primordial follicles compared to a control group with no mutations, consistent with a depleted ovarian reserve. How DNA damage repair proteins regulate follicle count is unknown, but as stated above, utilizing these models represent an excellent method of studying ovarian senescence and its relation to biological events inside the oocyte.

Third, other rare genetic progeroid (premature-ageing like) syndromes are all caused by mutations in DDR proteins, including but not limited to the RecQ helicases, required for DNA replication, repair and recombination, transcription coupled repair (*ERCC6*, *ERCC8*) and inter-crosslink repair (*FANCA*, *FANCC*, *FANCG*) (Schumacher *et al.*,

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2021). Hence, DNA damage is a central theme in the process of ageing, and oocytes are particularly susceptible to it due to long periods in a dormant state during which genome instability can accumulate. Altogether, the DDR pathway, as the acting sentinels of the genome, may represent the unifying link between oocyte quality and age.

It is still mysterious why mammals have evolved to generate large numbers of oocytes during oogenesis just to have massive amounts of attrition early in life. One possibility is that somehow the process of oogenesis itself creates oocytes that may contain DNA damage requiring attrition and quality control. Otherwise, it is hard to understand how widespread apoptosis in the ovary could be beneficial. This is especially perplexing given that oocyte chromosome segregation is error prone as well. The only way to reconcile the damaging and error prone processes of oogenesis and oocyte chromosome segregation with their essential functions in ensuring species continuity, is to assume it confers an evolutionary advantage. One speculation is that these inherently vulnerable processes are an evolutionary adaptation that makes them susceptible to physiological ageing. This would either allow females a post-reproductive phase where they can care for the next generation's progeny (i.e. the 'grandmother' hypothesis) or offset the morbidity risks of late-life reproduction (i.e. the 'mother' hypothesis); although both are debatable (Croft *et al.*, 2015).

Epigenetic age acceleration: opportunities and challenges for developing predictive tools for reproductive and post-reproductive ageing

Establishing a non-invasive tool that can measure the effect of anti-ageing interventions on female reproduction (reviewed in Tesarik *et al.* (2021)) or monitor the rate of ovarian reserve exhaustion under the influence of external insults and environmental toxicants would be of great value given the inaccessibility of ovarian tissue. In mice, an epigenetic clock based on 90 CpG methylation profiles from blood (Petkovich *et al.*, 2017) can predict biological age and the effects of environmental factors on longevity (e.g. caloric restriction). In humans, pan-tissue and blood-based epigenetic clocks have been developed as a biomarker of chronological age based on DNA cytosine-5′ methylation level measurements at specific CpGs (Hannum *et al.*, 2013; Horvath, 2013). Expanding on these DNA methylation clocks, estimators were developed to predict lifespan (time to death for all-cause mortality) after adjusting the regression model for risk factors and chronological age (Levine *et al.*, 2018; Lu *et al.*, 2019; McCrory *et al.*, 2021).

How do the epigenetic clocks perform in predicting ovarian age? The Horvath pan tissue epigenetic clock concept, which is based on estimating the relative methylation levels of 353 CpG islands, was implemented in whole blood cells to interrogate possible correlations between menopause and biological ageing as predicted by epigenetic age (Levine *et al.*, 2016). Meta-analysis on the blood methylation levels from three cohorts (WHI, InCHIANTI and PEG) uncovered a strong association between epigenetic age acceleration and earlier onset of menopause (Levine *et al.*, 2016). Menopause was also found to accelerate epigenetic ageing in blood, indicating that the associated endocrine changes during the post-reproductive period could affect epigenetic age of other tissues, thus inducing ageing (Levine *et al.*, 2016). The same concept was implemented to construct epigenetic clocks on human white blood cell and cumulus cell (CC) methylation profiles from a cohort of poor and good responders to ovarian stimulation (Hanson *et al.*, 2020). Blood epigenetic age acceleration estimates were statistically significant between poor and good responders when participants <38 years old were analysed but not when all samples were included in the analysis. There was no association between poor and good responders with the epigenetic age acceleration estimates on CC methylomes. Importantly, the CC methylome-based epigenetic clock did not associate with chronological age, which most likely can be attributed to the different mechanisms defining age in the follicular cells (oocytes and CCs).

Further work from the Horvath group, exploited bovine oocytes to test whether epigenetic age estimates from the blood methylome parallel those measured by the epigenetic clock in oocytes and whether bovine oocyte epigenetic age can be used to model human oocytes (Kordowitzki *et al.*, 2021). However, the correlation between DNA methylation ageing was low between oocytes and blood and could not be captured by the targeted array-based CpG methyl profiling (Kordowitzki *et al.*, 2021). It is also worth noting that single-cell sequencing in mouse oocytes, revealed that although global CpG methylation was modestly reduced in old compared to young oocytes, the characteristic oocyte methylation patterns and imprinted regions were unaffected by age (Castillo-Fernandez *et al.*, 2020). Therefore, a relationship between earlier age at menopause and accelerated epigenetic ageing, and whether the epigenetic clocks could predict the time to reproductive senescence is unresolved. It remains to be seen whether an epigenetic clock developed in one species can effectively be translated to another. A longitudinal study of epigenetic ageing before and after menopause would provide insights into the association of epigenetic age acceleration/deceleration with the age at the onset of menopause.

Given the differences between somatic and germline cells comprising a follicle, prior to implementing the statistical and mathematical concepts of the epigenetic clocks, comprehensive methylation studies to identify the differentially methylated regions (DMRs) that capture the effect of age in cell type-specific methylomes are needed. In this direction, Olsen and colleagues developed a mural granulosa cell (MGC) specific epigenetic clock (Granulosa Cell Clock), using 296 CpGs defined for this somatic ovarian cell type (Olsen *et al.*, 2020). The granulosa clock performed well in predicting MGC chronological age, in contrast to the 353 CpG pan-tissue clock. In line with the prediction of different methylation patterns between ovarian and non-ovarian somatic tissue, the age-associated MGC DMRs were not reproducible in leucocytes. Genes with strong age-associated MGC DMRs and a lack of association with non-ovarian somatic tissues, include *VTRNA2-1*, *AMH*, *ZFP57*, *PGF*, *GHSR*, *GHR* and *GAPDH*, all of which have important roles in folliculogenesis (summarized in Olsen *et al.* (2020)), suggesting that MGCs age differently to non-ovarian somatic cells in the human body. MGCs play a pivotal role in folliculogenesis and in interactions with the oocyte, therefore further knowledge about ageing of the MGC may provide important insights into ageing of the oocyte itself (Olsen *et al.*, 2020). Taken together, the findings of that study,

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strengthen the hypothesis that MGCs age differently to non-ovarian somatic cells in humans.

Single-cell transcriptomic studies emphasize the role of understanding tissue heterogeneity in regulating functional heterogeneity through diverse cellular programs in the ovary (Fan *et al.*, 2019; Wagner *et al.*, 2020). Further dissection of these programs at temporal and spatial resolution across developmental and follicular stages and their association with methylome changes will (i) advance our understanding of ovarian remodelling in the context of ageing and (ii) create an opportunity for the development of a predictive tool based on ovarian DMRs. It would still have to be associated with peripheral blood signatures to render it non-invasive.

Conclusions

A plethora of physiological and environmental factors impact the female reproductive life span but, according to our current knowledge, they all converge in the determination of the ovarian reserve size and the rate of its depletion through DNA damage repair. Recent large scale genomic studies have started to elucidate how genetic variation affects variation in the ANM and importantly has informed functional experiments targeting specific molecular players for pharmacological or life-style treatments. Following the example of other complex traits, larger cohorts are anticipated to expand the associated loci. However, more tissue- and context-specific transcriptome and methylome datasets need to be created to enable informative QTL discovery and lead functional experiments. In parallel, the bridge between comparative cell biology and genomics needs to become stronger to expedite discovery in appropriate animal models.

Data availability

This study only contains a narrative review of existing data. No new data were generated by the authors.

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Authors' roles

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Conflict of interest

The authors declare they have no conflicts of interest.

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