Germline passage of mitochondria: quantitative considerations and possible embryological sequelae

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Using a semi-quantitative review of published electron micrographs, we have explored the passage of mitochondria from one generation to the next through the cytoplasm of the human female germ cell. We propose a testable hypothesis that the mitochondria of the germline are persistently 'haploid' (effectively carrying just one mitochondrial chromosome per organelle). For mitochondria, the passage through germ cell differentiation, oogenesis, follicle formation and loss could constitute a restriction/amplification/constraint event of a type previously demonstrated for asexual purification and refinement of a non-recombining genome. At the restriction event (or 'bottleneck') in the human primordial germ cell, which differentiates in embryos after gastrulation, there appear to be <10 mitochondria per cell. From ~100 or so such cells, a population of $\geq 7 \times 10^6$ oogonia and primary oocytes is produced in the fetal ovaries during mid-gestation, with mitochondria numbering up to 10 000 per cell, implying a massive amplification of the mitochondrial genome. A further 10-fold or greater increase in mitochondrial numbers per oocyte occurs during adult follicular growth and development, as resting primordial follicles develop to pre-ovulatory maturity. So few are the numbers of oocytes that fertilize and successfully cleave to form an embryo of the new generation, that biologists have long suspected that a competitive constraint lies behind the generational completion of this genetic cycle. I propose that maintaining the integrity of mitochondrial inheritance is such a strong evolutionary imperative that features of ovarian follicular formation, function, and loss could be expected to have been primarily adapted to this special purpose. To extend the hypothesis further, the imperative of maintaining mitochondrial genomic integrity in a population could explain why women normally become sterile a number of years before there is depletion of ovarian follicles and endocrine ovarian failure (i.e. why there is 'an oopause' preceding the menopause). Plausible explanations might also follow for several well-known and puzzling reproductive difficulties, including recurrent miscarriage, unexplained infertility, and persistent failure of IVF embryos to cleave or to implant. Current experimental laboratory manoeuvres that might circumvent mitochondrial shortcomings (such as cytoplasmic transfusion and karyoplast exchange) are examined and possible clinical hazards identified.

Introduction

A number of important questions concerning ovarian function and dysfunction remain in need of satisfactory explanation: (i) why is it that, in probably all mammalian species, the complement of female gametes is produced early in the animal's life, before the attainment of sexual maturity?; (ii) why is there such a high rate of attrition of ova in mammals, specifically in humans?; (iii) could there be a purpose for the mitotic frenzy of ovarian follicular cells during the process of follicle growth beyond the secretion of oestradiol and the release of an oocyte surrounded by mucus that is sticky for the fimbrial end of the Fallopian tube?; (iv) what causes the deterioration of oocyte competence observed in women in their late 30s, a decade or so before final follicular depletion at the menopause?; and (v) might mitochondrial dysfunction be the ultimate cause of some disorders of ovulation, unexplained infertility, and recurrent miscarriage?

Whether a mitochondrial genomic perspective could provide answers to these questions constitutes the subject of this paper. The mitochondrial quantifications have been previously published (Jansen and De Boer, 1998).

Mitochondria during oogenesis

Throughout oogenesis and early embryogenesis, mitochondria in germ cells differ in appearance from somatic cell mitochondria. Morphologically, except during presumed times of high mitochondrial multiplication close to the nucleus, mitochondria in female germ cells assume unique spherical profiles (Smith and Alcivar, 1993). It is known that mature mammalian oocytes have about the same number of mitochondria as they have mitochondrial (mt) DNA circles: they are effectively haploid, containing mostly just one mtDNA molecule per organelle (Pikó and Matsumoto, 1976; Michaels *et al.*, 1982; Pikó and Taylor, 1987). An elongated, typically somatic cell mitochondrial morphology (Smith and Alcivar, 1993) and normal replication patterns (see Larsson *et al.*, 1998, for consequences if replication fails) are established together, after implantation (see also Shepard *et al.*, 2000).

We have used previously published electron micrographs of the various stages of human oogenesis to estimate the number of mitochondria at each stage of human oogenesis (Figure 1) (Jansen and De Boer, 1998). The fewest mitochondrial profiles per germ cell are seen in the earliest primordial germ cells (PGCs), still in the endoderm of the yolk sac of 3 week embryos, with about four or five visible profiles per cell, predicting a mitochondrial number of <10 (Figure 2). This number of mitochondria might be the lowest that occurs in the transmission of cytoplasm from one human generation to the next: a restriction event to qualify for the first step in reversing Muller's ratchet (see Jansen, 2000). It cannot be ruled out that an even lower number might have occurred in the embryonic germ cell line at gastrulation, a week earlier.

In their consideration of a numeric restriction event in mitochondrial transmission, Michaels *et al.* (1982) assumed that embryonic cells prior to primordial germ line differentiation would have a general somatic cell (i.e. polyploid) mtDNA copy number per organelle; they concluded that the reduction (and hence the location of the bottleneck) occurs later in oogenesis. No direct measurements of mtDNA copy number have been made on PGCs. It should be noted, however, that in species as diverse as the frog (Dawid, 1972; El Meziane *et al.*, 1989) and the mouse (Ebert *et al.*, 1988; Pikó and Chase, 1993) no
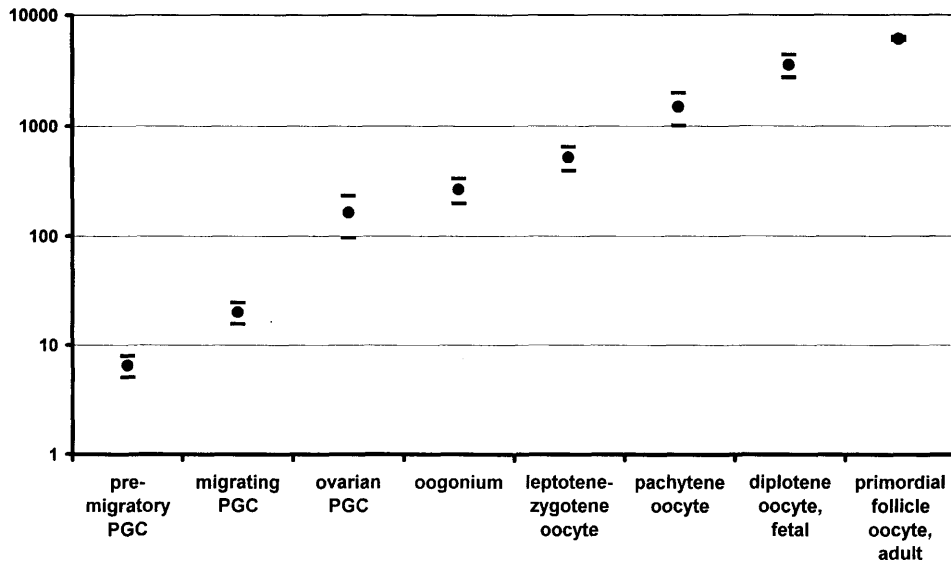
Figure 1. Log-scale estimate of approximate mitochondrial number per female human germ cell through oogenesis and folliculogenesis, determined by a volumetric transformation of published, single section, electron micrographs. PCG, primordial germ cell (reproduced from Jansen and De Boer, 1998, with permission).
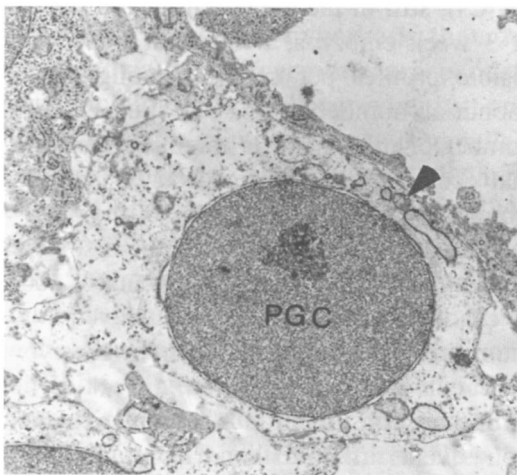

Figure 2. Human fetal primordial germ cell (PGC) in the yolk sac endoderm prior to active migration to the genital ridge, showing a paucity of cytoplasmic organelles, low mitochondrial number, and abundant glycogen. Original magnification ×12 000 (reproduced from Makabe and Motta, 1989, with permission).

new mtDNA is synthesized during embryogenesis until approximately the stage of gastrulation. If this is true also in humans, in which gastrulation occurs in the day 15 embryo, there is a period of at most only a few days during which the mitochondrial genome of the germ cell line might become polyploid prior to the germ cells' migration to the developing gonadal ridges. It is our hypothesis that the mitochondria in cells of the germ line do not become polyploid at all, but remain essentially haploid from generation to generation (Jansen and De Boer, 1998).

Already during PGC migration (a time when, on morphological evidence, glycogen is utilized by the PGCs), mitochondrial numbers increase, and hence by implication mtDNA replication (i.e. amplification) has begun. After differentiation into oogonia, the mean mitochondrial number per cell is ~200, and there is a qualitative change in the form of the inner mitochondrial membranes (the cristae) from a villiform pattern to a sparse, arched form that will also typify the mitochondria through nuclear meiosis (Figure 3) (Lanzavecchia and Mangioni, 1964; Baker and Franchi, 1967; Wartenberg, 1974; Gondos, 1987; Gosden, 1995). Such an appearance, with separation of inner mitochondrial membranes, could reflect oxidative inactivity (Shepard, 1998) and protection of the maternal mtDNA from the
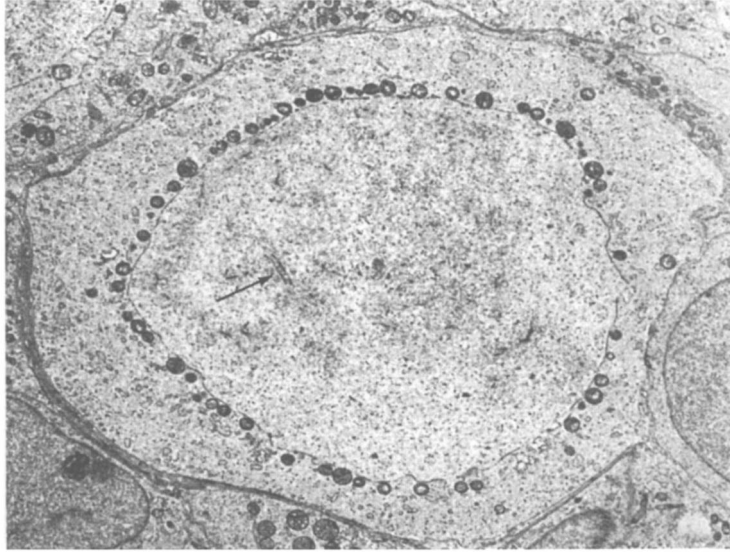
Figure 3. Human fetal pachytene primary oocyte, with an increase in mitochondrial number taking place close to the nucleus, which demonstrates synaptonemal complexes (arrow) indicative of nuclear chromosomal crossover. Original magnification ×5000 (reproduced from Gondos *et al.*, 1986, with permission).

mutagenic stress of reactive oxygen species (Allen, 1996). The replication of germ cells that takes place over the next few months of fetal development sees an increase in germ cell numbers (Baker, 1963) to ~7×10^6 (an increase of 2^{18} over the yolk sac PGC number), and an implied increase in total germ cell mitochondria to ~35 billion (an increase of 2^{29} over the assumed yolk sac germ line mitochondrial total). During this explosive amplification in numbers, mitochondria mostly retain their spherical profile.

In pachytene of the first meiotic division, when synaptonemal complexes form in the nuclei of the developing oocytes to allow genetic recombination to take place in the diploid nuclear genome, human mitochondria replicate while located close to the nucleus (Figure 3) (Lanzavecchia and Mangioni, 1964; Baker and Franchi, 1967; Gondos *et al.*, 1986), giving cross-sections of human pachytene oocytes the appearance of a necklace of mitochondria, a 'mitochondrial crown', as it has been called in amphibian oocytes at this stage of germ cell development (Mignotte *et al.*,

1987). Recent data imply that mitochondrial DNA replication in somatic cells is preferentially located close to the nucleus (Davis and Clayton, 1996). Later, in diplotene, among the surviving oocytes that have formed primordial follicles, the mitochondria are found within lamellations of endoplasmic reticulum parallel with and to one side of the nucleus (Figure 4) (Lanzavecchia and Mangioni, 1964). Studies of *Xenopus* (frog) pachytene and diplotene oocytes show a similar mitochondrial arrangement (Callen *et al.*, 1980; Tourte *et al.*, 1984): incorporation of radioactive thymidine reveals that mitochondria segregate into two populations at this stage of oogenesis in frogs, with one population close to the nucleus, actively replicating mtDNA and building up most of the stock of the mitochondria for the full-grown oocyte, while the second population, in which DNA replication has stopped, occupies a 'mitochondrial mass' in the vegetal pole, which constitutes the germ plasm (cytoplasm destined for the germ cells) in this species.

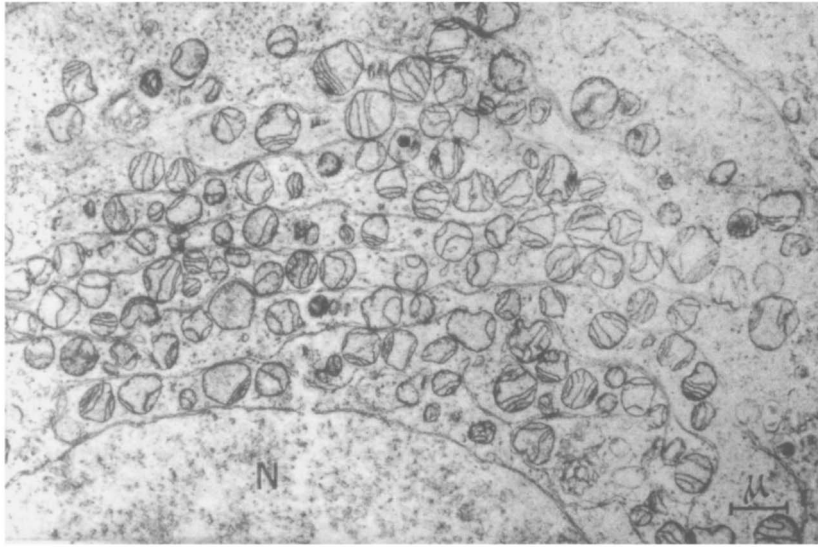In humans at birth, the number of oocytes

Figure 4. Human diplotene primary oocyte, with mitochondrial accumulation within lamellations of endoplasmic reticulum, to one side of the nucleus (N). Original magnification ×9000 (reproduced from Lanzavecchia and Mangioni, 1964, with permission).

(all now encompassed within primordial follicles) has fallen to ~2×10^6, by menarche to ~300 000 (Baker, 1963), and by the age at which there is a well-known decrease in oocyte reproductive competence (probably in the range 35–45 years) (Serhal and Craft, 1989; Jansen, 1995; Faber *et al.*, 1997) to ~25 000 (Faddy *et al.*, 1992), after which there could be an exponential acceleration in numeric decline (Faddy *et al.*, 1992; but see Leidy *et al.*, 1998, for a cogent contrary interpretation). Although proof has been lacking, the purge of oocytes that takes place before and after birth is widely suspected of being a mechanism of ridding the germ line of genetically inferior eggs. If so, there could be the opportunity to purge defective mitochondrial genomes.

With growth of the oocyte during resumed follicular development, the mean mitochondrial number in human oocytes appears to increase from ~10 000 in the primordial follicular oocyte to around 300 000–400 000 in the mature oocyte in metaphase II (according to approximations derived from published micrographs, see Figure 1). The mitochondria away from the perinuclear replicating zones remain spherical in form, with sparse cristae, but accumulate electron-dense material in the mitochondrial matrix (Hertig and Adams, 1967; Sathananthan *et al.*, 1986), an appearance that persists after fertilization until the 2-cell stage. With oocyte maturity at ovulation, mitochondrial multiplication (Taylor and Pikó, 1995) and mtDNA replication (Pikó and Matsumoto, 1976; Ebert *et al.*, 1988; Larsson *et al.*, 1998) stop (at least in the mouse, but replication also stops in the frog embryo, so presumably also in the human), not to be resumed until after implantation, at about the time of gastrulation.

When cleavage commences after fertilization, the matrix of the mitochondria becomes less dense and cristae increase in abundance (Dvorak and Tesarik, 1985; Sathananthan *et al.*, 1993; Motta, 1995), while mitochondrial transcription resumes (Pikó and Taylor, 1987) and pyruvate (which enters the mitochondrial citric acid cycle for combustion) becomes the embryo's preferred energy substrate over glucose (Gardner and Sakkas,

1993). This more active appearance of the mitochondria is mirrored in the nucleus by the de-compaction of the nucleolus, indicating simultaneous resumption also of nuclear transcription. Thus, it is plausible that the decreasing density of mitochondrial matrix contents at this time represents incorporation of previously imported, maternal genome-derived protein components of respiratory chain enzyme complexes, stored in anticipation of mitochondrial transcription and translation of the remaining protein subunits, and incorporation of complete complexes into the new inner mitochondrial membrane.

Germline mitochondrial DNA recombination?

Just as Chao's viruses recovered genomic fitness faster with a relatively simple form of recombination (segment reassortment) added to their predominant reliance on non-sexual means to reverse Muller's ratchet (Chao *et al.*, 1997; Jansen, 2000), so it is plausible that mitochondria also rely to a degree on both non-recombinant and recombinant strategies. The possibility of reciprocal recombination occurring between female and male germ cell mitochondria has classically been considered doubtful (Hauswirth and Laipis, 1985), the evidence against it being that nucleotide sequence differences separated by ~3000 bp are consistently coinherited (Olivo *et al.*, 1983). But this evidence contradicts recombination between interorganismal mtDNA genomes: it does not argue against intra-organismal recombination. Mitochondria in vertebrate oocytes have the enzymatic machinery to carry out homologous recombination, namely excision repair involving DNA polymerase γ (Ryoji *et al.*, 1996) and possibly mitochondrial DNA ligase (at least as indicated by its isolation from homogenized *Xenopus* ovaries) (Pinz and Bogenhagen, 1998). Somatic cell mitochondrial protein

extracts are able to catalyse recombination of plasmid DNA substrates *in vitro* (Thyagarajan *et al.*, 1996), and it is possible that mitochondrial deletions in somatic cells occur as a result of prior illegitimate recombination (see Howell, 1997, for review).

Embryonic survival and a new generation, or failure

The observation that mitochondrial DNA synthesis is suspended until gastrulation in species as diverse as the frog (El Meziane *et al.*, 1989) and the mouse (Larsson *et al.*, 1998), vertebrate lines that diverged >400 million years ago, implies that there could be a universally important biological principle behind the suspension. Gastrulation is soon followed by sequestration of the first PGCs in the posterior wall of the yolk sac.

If true also in humans, the morphological data summarized quantitatively in Figure 1 places the major site for the mitochondrial population bottleneck at or before the stage of the pre-migratory PGC, with a mitochondrial number (hence by implication a mtDNA copy number) of probably <10 per germ-line cell. Depending on how and when mtDNA synthesis and replication restarts in the germ cell line (presumably from a juxtanuclear mitochondrial subpopulation), it is plausible that there might be a further restriction in the number of founding mitochondrial chromosomes, with a minimum in some circumstances (perhaps when the genome is under special challenge from one or more mutations) (Blok *et al.*, 1997) of a single founding mitochondrial genome.

The gap between resumption of transcription (after the 2-cell stage in the mouse) (Pikó and Taylor, 1987; Taylor and Pikó, 1995) and new mtDNA synthesis (at about the stage of gastrulation) means that the quantitative as well as the qualitative endowment of mtDNA species to the conceptus will come under strain

as the extant mitochondria are diluted and partitioned into multiplying daughter blastomeres. Recurrent miscarriage can be an early feature of Wilson's disease (preceding clinical liver disease by many years; Schagen van Leeuwen *et al.*, 1991), in which a faulty copper-transporting protein gene-product located in mitochondria results in intramitochondrial accumulation of copper, causing oxidative damage to mtDNA (Mansouri *et al.*, 1997). Elucidating the nature of the normal permissive stimulus for resumption of mtDNA synthesis (and the requirements for successful resumption) could yield insights into the molecular aetiology of early pregnancy failure.

Reversing Muller's ratchet: preserving or evolving the mitochondrial genome

For entropy and Muller's ratchet to be not just stopped but reversed (i.e. for there to be an improvement with time in a genome reproducing asexually) it is not enough to have mere winners at the end of a restriction/amplification/constraint cycle. The inheritance of potentially harmful human mtDNA point mutations (such as those behind the maternally inherited mitochondrial neuromuscular dystrophies MERFF, MELAS and Leigh's syndrome) from one generation to the next (Holme *et al.*, 1995) shows that the restriction/amplification/constraint cycle demonstrated above, with or without intraorganismal mtDNA recombination should it occur, is not in itself enough to prevent a deleterious drift of mtDNA along with a general decline in cytoplasmic health among the individual's descendants and hence among the general population (and eventually the species). The surviving genome might still show a slow degradation over time. There still needs to be a way of also preventing less pathological, but nevertheless deleterious, mutations from becoming fixed.

For free-ranging asexually reproducing organisms, a fluctuating environment, including competition with other organisms, will be enough to make sure that degrading genomes will become extinct during times of environmental deterioration, leaving the fittest to survive and evolve. However, for the host of a non-autonomous endosymbiont such as a mitochondrion this will not be enough. The host species will be in trouble if it is at the mercy of its parasitic endosymbiont long before the endosymbiont itself is threatened. It is therefore probable that the host species will have evolved a strategy for putting a stop to mitochondrial genomic degradation more satisfactory than high adult or even childhood mortality. I propose that a physiological state of sterility well before menopause could not only be mitochondrially based but could constitute a strategy for slowing Muller's ratchet.

It has been argued (Lynch *et al.*, 1993) that weakened mitochondrial genomes should not just be accepted by the host, that they need to have their suboptimal effects magnified to the extent that they will be recognized and readily eliminated from the population; i.e. that 'the extreme longevity of organelle lineages may be due not to their invulnerability to mutations and DNA damage, but to their extreme sensitivity to such effects'. Experience with the biochemical and clinical effect of mtDNA mutations in somatic tissues is now considerable (Brown and Wallace, 1994), if still confused by the polyploidal, compensated status of the somatic cell mitochondrial genome and the plastic nature of organelle fusion and fission. Nonetheless once a threshold of mitochondrial mutations is reached, the phenotypic consequences are typically inexorable and disastrous. Lynch *et al.* (1993) list the underlying molecular reasons for the unmitigated sequelae: proofreading and excisional repair genes have been lost; transfer RNA and ribosomal RNA genes, present in multiple copies in the nucleus, are single and vulnerable in mitochondria; mutagenic conditions are poten-

tially harsh and punishing, with excess superoxide radicles able to further disrupt the genotypic and hence phenotypic integrity of oxidative phosphorylation, generating more free radicles and a runaway process of accelerated mutation ('mutational meltdown'); and deleted mtDNA circles have a propensity to replicate faster than full circles, driving them to fixation and to the death ·of the host. Mitochondrial abnormalities are also highly leveraged clinically: disease occurs and leads to death of the organism well before tissues are critically deprived of ATP and widespread cell death occurs (E.A.Schon, Department of Neurology, Columbia University, New York); and mitochondria are intimately associated with pathways of apoptosis that lead to programmed cell death *in situ* (including, in the case of the ovaries, follicular atresia and oocyte death) (Tilly, 1996; Perez, *et al.*, 1997).

If these leveraged processes operate among haploid mitochondria of a germ line they could be just as tough in effect, if different in detail. In the case of the germ line, the ultimate test of the mitochondria for the new generation would be during the time between the onset of cleavage and successful implantation and gastrulation, when sequestration of a new germ line occurs. The mechanism for this final test of mitochondrial genomic integrity could be as simple as the constitutional enforcement of a delay in new mtDNA synthesis. This would ensure the demise of a conceptus that did not possess a sufficient number of normal mitochondrial genomic species. I further examine below what the expected clinical consequences of such an evolutionary device could be expected to be.

On a population level it is apparent that the mitochondrial genome does gradually deteriorate, with replacement of conserved sequences by more or less silent polymorphisms among the coding genes more prevalent within species than between species (Nachman, 1998), including the different

hominoids (Hasegawa *et al.*, 1998). Muller's ratchet 'clicks', despite the opposing safeguards discussed above. It has been estimated, however, that the resultant reduction of fitness from the accumulation of mutations is not likely to imperil many species on time scales of much less than a million years (Lynch and Blanchard, 1998), so this ultra-long term phenomenon can be ignored clinically.

In the shorter term of children or grandchildren, an improvement in mitochondrial genome quality ought to be possible from one generation to the next: the 'clock' of mitochondrial degradation that every adult animal accrues with age is 'reset' with every new generation.

Folliculogenesis and competition between follicles and oocytes

Of ~7 $\times 10^6$ germ cells that form in the ovaries (see above), at most a few hundred will ovulate. In the span of 4 weeks (every ovarian cycle during the reproductive years), tens or hundreds of follicles start their growth from the resting primordial state. Yet in most circumstances just one follicle each month presents its oocyte to be fertilized. The observer could reasonably suspect that he or she is witnessing a competition: waves of folliculogenesis and waves of atresia, with a very small number of winners (Krakauer and Mira, 1999).

Yet this is essentially the extent of the evidence that competition between ovarian follicles and between oocytes actually takes place. The evidence is circumstantial. There is still no compelling theory to explain even the initiation of a particular follicle's growth and development, let alone an indication of what it is that distinguishes follicles that can make it through to ovulation from those that cannot. The endocrine tide of fluctuating levels of follicle stimulating hormone and the once-a-month event of a LH surge is only the test

for the follicles: it does not explain the basis of differentiation between the candidates.

Ovarian folliculogenesis (and the opportunity for follicular atresia) is phylogenetically ancient, with junctional complexes between follicle cells and oocytes being found in developing follicles not only in all vertebrate classes, but also in the echinoderms (such as the sea urchins), which diverged from the chordate line >600 million years ago (Beijnink, *et al.*, 1984). So it could be that whatever it is that distinguishes some follicles over others will also be biochemically ancient and fundamental. The ability of follicles to express the means of generating energy, perhaps in the form of ATP, ought to be a strong contender. Follicles win by growing efficiently. They grow through the mitosis of their constituent granulosa cells. Meanwhile the oocyte nucleus rests in prophase of meiosis I, as it has since fetal life. Its mitotic test (should it return to diploidy through fertilization) comes after the follicle is spent, manifesting as cleavage, blastulation, gastrulation, and so on. Could the mitotic competence of the granulosa cells somehow be a direct indicator of what the oocyte's genomic integrity, what its metabolic strength, and what its mitotic potential will be after syngamy? So inextricably linked is the fate of an oocyte to the fate of its follicle that it would be reasonable to put the hypothesis that it is the metabolic potential of the oocytes (i.e. the genomic integrity of oocyte mitochondria) that is being tested by a metabolic competition between follicles.

At this stage it is speculation, but for illustrative purposes there *are* plausible mechanisms linking oocyte and follicle cell metabolic competence, mechanisms that would confer further purpose upon the intimate cytoplasmic contact granulosa cell processes have with oocytic cytoplasm via gap junctions permeable to small soluble molecules (Buccione *et al.*, 1990; Grazul-

Bilska *et al.*, 1997). Such junctional contact is absolutely required for oocyte growth (Eppig, 1979), and mediates the transfer of substances inhibitory to meiosis from granulosa cells to the oocyte (Tsafriri *et al.*, 1982), but also transmits from ooplasm to adjacent granulosa cells soluble substances that cause differentiation of the developing antral follicle (Li and Mather, 1997) and, later, of cumulus cells (Eppig *et al.*, 1997). The oocyte's ATP content correlates significantly with the developmental potential of the embryo after fertilization *in vitro* (Van Blerkom *et al.*, 1995), but it is uncertain whether the ATP is generated by the oocyte, by the granulosa (follicles with poorly competent oocytes are hypoxic) (Van Blerkom *et al.*, 1997), or by both, as gap junctions are freely permeable to nucleotides, including the intracellular signalling nucleotide cyclic AMP (Lawrence *et al.*, 1978). At this point, it may at least be accepted that metabolic traffic between granulosa cell cytoplasm and oocyte cytoplasm will be two-way.

If the mitochondrial mutational load of at least some ovulated oocytes turns out to be a significant constraint on the development of many superovulated and recovered oocytes in clinical IVF programmes (Keefe *et al.*, 1995), then the ability of the mitochondrial genome to restore itself to homoplasmic normality for virtually every newborn (Koehler *et al.*, 1991) (if this assumption is true: data are limited) looks like a remarkable physiological achievement. I suggested above that physiological sterility well before functional follicular depletion at the menopause, the phenomenon we have called the oopause (Jansen and De Boer, 1998), could be an essential component of the required survival strategy of the haploid mitochondrial genome, and could be based on the simple (and evolutionarily conserved) device of postponement of new mtDNA synthesis in the new host organism until after gastrulation. Too few normal mtDNA species

in the fertilized oocyte and the ovum would be permanently blighted.

As an end-point, however, female sterility will not be a favoured option for survival of a species if it happens too readily. In evolutionary terms, one would suppose that this certainty would surely have caused immense pressure to have devolved back on the processes of oogenesis and folliculogenesis, oocyte competition, and competition between follicles to correct genetic inferiority. I propose that the imperative of conserving the mitochondrial genome could be a fundamental reason why these structures and this system developed.

The oopause and other enigmatic manifestations of reproductive dysfunction

The stringent bottleneck through which the mitochondrial genome passes, and by which mitochondrial DNA is refined and fixed from one generation to the next, could thus be the same bottleneck that permits only relatively young eggs with healthy cytoplasm to have a high chance of implanting successfully after fertilization *in vivo* or *in vitro* (Jansen, 1995; Faber *et al.*, 1997). At what age can sterility from such a mechanism be expected?

The age at which that the oopausal transition can become important, at least commercially in the United States, is as early as ~33 years, after which donated eggs begin to produce progressively lower rates of pregnacy, thus tending to disqualify recruitment as a paid donor in US donor egg programmes (Faber *et al.*, 1997). A decrease in fecundability is seen earlier than this, being evident after the age of 30 years, but it is accelerated after 35 and it falls sharply after 40. From 42–44 years, depending partly on the quality of the laboratory, pregnancy with IVF becomes extremely rare (although natural successful conception is still reported among 1 in 10

women at 45 in societies where contraceptives are minimal (Lorimer, cited by Guttmacher, 1956). The average age at which physiological sterility occurs in women can be estimated from populations of women with a low natural rate of environmentally induced sterility and who do not practise contraception, such as the North American Hutterite communities. Hutterite women in the Dakotas, Montana, Alberta and Manitoba have a median age at marriage of 22 and a total primary sterility rate of only 3.4%; they conceive an average of 10.4 times in a reproductive lifetime (Guttmacher, 1956), and their median age at last conception is ~40.5 years (Tietze, 1957), which is 10 years earlier than the median age at menopause among Western women (Walsh, 1978).

The traditional explanation for declining oocyte health and embryonic potential with female age has been an increasing prevalence of chromosomal aneuploidy. Does this explanation challenge a mitochondrial explanation? Many authors (see Schon *et al.*, 2000) speculate that it might not.

First, it should be remembered that although the prevalence of aneuploidy rises among conceptions with age, this prevalence always falls far short of 100%, irrespective of how much fertility falls as female age rises. Even at the age of 45 years, when the risk of miscarriage is >50% of conceptions, a large minority of miscarriages have a normal karyotype, and no other diagnosable cause can be found. Second, aneuploidy itself results from defective chromosomal-spindle attachment or function: mitochondria normally aggregate around the metaphase spindle in mouse oocytes, implying a local need for ATP production in spindle formation and molecular motor function (Van Blerkom and Runner, 1984; Li and Fan, 1998); oxidative stress has been causally linked to meiotic nondisjunction (Tarín *et al.*, 1998); defective spindles in published electron micrographs of human

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oocytes have been noted to have comparatively few mitochondria in their vicinity (Sathananthan *et al.*, 1986); and a mutation that prevents mitochondrial binding to meiotic spindles in the fruit-fly *Drosophila* leads to sterility (Basu and Li, 1998). In other words, it might be that mitochondrial dysfunction in oocytes is more fundamental than, and possibly even causes, aneuploidy.

From clinical observations (R.P.S.Jansen, unpublished), a woman at the age of the oopausal transition can have repeated karyotypically normal miscarriages despite successfully having had several children at an earlier age with the same partner, the final outcome by the late-30s to mid-40s being otherwise unexplained infertility. Larsson *et al.* (1998) showed that transgenic mouse embryos homozygous for disruption of mitochondrial transcription factor A (a nuclear gene product essential for initiation of mitochondrial DNA replication—see Clayton, 2000) and transferred to normal mothers will proceed through implantation and gastrulation, before ATP production fails and embryonic resorption occurs. Gastrulation is the point at which dilution of the oocyte's original mitochondrial complement falls below that required to sustain further development (see above). An equivalent embryonic mitochondrial failure in humans would be expected to present at or before an equivalent time, with failure of the embryonic mass to proceed, namely a *blighted ovum* (the classical term for an anembryonic pregnancy sac). Looking for mitochondrial genetic causes for sporadic and recurrent miscarriages at any age could be a fruitful area for research.

A genomic insufficiency of mtDNA in the oocyte might account for progressively severe reproductive dysfunction at any stage before new mitochondria are due to be produced, both with natural conception and with IVF, and without necessarily incurring aneuploidy (Lower *et al.*, 1991). Starting with early

miscarriages, biochemical pregnancies (subclinical miscarriages) could be expected to follow increasingly severe quantitative or qualitative mitochondrial insufficiency, in turn followed by unexplained implantation failure (unexplained infertility or failure to conceive with IVF/embryo transfer), then slow or arrested cleavage *in vitro*, fertilization failure, and finally a failure even of ordered pre-ovulatory follicular growth, if follicular cell mitosis can indeed be linked to oocyte health.

In summary, as well as determining what could be called an oopause a decade or so before menopause, I propose that there could be a mitochondrial basis behind a number of previously inexplicable reproductive difficulties, such as unexplained infertility and recurrent miscarriage. Furthermore, poorly selected oocytes from superovulated ovaries or imperfect culture conditions in IVF laboratories (adding an iatrogenic component to the damage delivered by imperfect oocyte mitochondria) could explain the discrepancy that whereas 10% of last conceptions can occur at > 45 years in natural circumstances (Tietze, 1957), non-donor oocyte IVF conceptions become exceedingly rare after 42 or 43 years. Preliminary quantification of mtDNA from superovulated secondary oocytes in women of different ages indicate that there could be a trend towards lower counts with increasing age (De Boer *et al.*, 1999). If so, this could be a widespread phenomenon among female vertebrates, as a similar age-related decline in oocyte mtDNA has been described in frogs (Callen *et al.*, 1980).

Physiological prevention of pregnancy prior to oocyte depletion could be the ultimate constraint that prevents degraded mitochondrial genomes entering or persisting in the reproducing population. According to this hypothesis, the mitochondrial genome, not exposed to fluctuating external environments for natural selection, is instead subjected to a different yet tough test of compliance: the

need for there to be numerically sufficient normally functioning mtDNA circles in an oocyte for effective survival of the zygote for 2 weeks before new mtDNA synthesis is permitted. If normal mitochondrial DNA forms are too few in number to outstrip the dilution that divides a fixed number of forms into the multiplying cells of the conceptus, the conceptus ought to stop developing and pregnancy will then fail. If ageing oocytes carry fewer non-degraded mitochondrial genomes, this means that the device of postponing mitochondrial DNA replication until gastrulation has occurred could substantially limit intergenerational mitochondrial genomic deterioration, albeit at the cost of sterility well before final depletion of follicles and oocytes. For abnormal mitochondrial genomes that are able to slip through whatever selection process is going on (such as those with certain single point mutations), a fitness decline with reduced reproductive capacity in the new host organism is predictable, ranging clinically from the mild to the catastrophically severe (Holme *et al.*, 1995; Ferlin *et al.*, 1997). Both of these clinical phenomena, mitochondrially dependent physiological sterility and the spectre of mitochondrial disease among the offspring, could turn out to be important in the management of subfertility and sterility in older women.

Implications for therapeutic IVF

What might be done therapeutically for women facing physiological but unanticipated sterility from a mitochondrially determined oopausal transition? Aside from whole egg donation by a younger woman, there are two obvious strategies for overcoming ooplasmic ageing, at least while fertilization and normal pronuclear formation *in vitro* is still possible. There has already been clinical experience with both, so it is worth extending the theoretical considerations derived above to these manoeuvres.

Cytoplasmic replacement by karyoplast exchange

Replacing the bulk of the mitochondrial genome could be achieved by swapping the pronuclei of a fertilized oocyte from 'old' cytoplasm to the enucleated fertilized oocyte of a donor (the micromanipulations of the karyoplasts involved have been shown to be workable in animal studies; Smith and Alcivar, 1993) or by exchanging the nucleus of a germinal vesicle stage oocyte prior to maturation. These could be options worth exploring, not just for women in the oopausal transition but also for women with clinically important mtDNA mutations. A number of studies that highlight practical problems have been performed.

First, ooplasmic mitochondria aggregate around pronuclei about to undergo syngamy (Van Blerkom and Runner, 1984), so it is virtually impossible not to include a substantial population of mitochondria with the taking of a karyoplast from a pronuclear zygote, removing useful mitochondria from the recipient cytoplasm (the donated oocyte) and transferring questionable mitochondria along with the infertile couple's pronuclei into the normal remaining recipient cytoplasm. Studies in mice have indicated that such karyoplast-associated mitochondria might be favoured, even during cleavage (Meirelles and Smith, 1998).

Second, there are data implicating interference with essential nuclear–mitochondrial dialogue to explain decreased cytoplasmic performance and increased failure to form blastocysts among reconstructed zygotes made from interspecific crosses among mice (Smith and Alcivar, 1993; Nagao *et al.*, 1998). The data are difficult to interpret. There are also indications that there could be considerable tolerance in nuclear–mitochondrial dialogue in early embryos, even across species (Dominko *et al.*, 1999). Intraspecific transfer of calf

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embryo nuclei from separated early morula blastomeres into enucleated secondary oocytes has been associated with high variability in body size among offspring (Gärtner et al., 1998) – perhaps an unsurprising consequence, given the greatly different state of nuclear and mitochondrial transcription between these two stages. Whether difficulties in nuclear-mitochondrial communication will accompany intraspecific, possibly intrafamilial exchanges of comparably staged human pronuclear embryos or 2-cell embryos awaits further research.

Clinical experiments involving germinal vesicle-stage nuclear transfers in humans have not proven successful to date (Zhang et al., 1998), but the main difficulties could lie with the in-vitro maturation these oocytes have required.

Cytoplasmic transfusion or cytoplast transplantation

Without its having been stated explicitly, mitochondrial supplementation could be the reason behind the birth of infants after transfer of anucleate donor oocyte cytoplasm to recipient secondary oocytes reported in a group of older infertile women (Cohen et al., 1997, 1998). Women who were aged >35 years were treated; women with a record of good numbers of eggs retrieved, but with multiple unsuccessful embryo transfers after IVF: the embryos were deemed poor on morphological criteria. Cohen et al. found that normal fertilization was higher after direct injection of 7–14% of ooplasmic volume at intracytoplasmic sperm insertion (which produced a 63% fertilization rate, improved embryo morphology and several pregnancies) than after electrofusion of donor ooplasts 40–90 min before ICSI (which produced a 23% fertilization rate, pronuclear anomalies, no correction of embryo morphology, and no pregnancies). At least 10

delivered or ongoing pregnancies have now been reported (see Barritt et al., 2000).

Nuclear and mtDNA fingerprinting profiles of the donor and recipient of such cytoplasmic transfusion for oocytes were studied at amniocentesis in the first ongoing oocytoplasmic transfusion pregnancy, later delivered at term, and no donor profiles for the donor genome (nuclear or mitochondrial) were detected by PCR and sequencing (Cohen et al., 1997; 1998). The infant was clearly the daughter of her parents' nuclear genomes. Donor and recipient mtDNA differed at positions nt16219 and nt16311 (part of the small, non-coding, D-loop region of the genome, where polymorphisms are frequent) and an examination was undertaken that excluded any significant survival of donated mtDNA. It would appear from these limited data that the donated mitochondria were perhaps sufficient to assist embryonic survival beyond implantation and gastrulation but, at least in this case, then did not take part in the subsequent resumption of mitochondrial replication.

It is plausible that children born after cytoplasmic transfusion of secondary oocytes will be born with a mitochondrial genome of a level of fitness that would normally not be compatible with survival. This need not be because of inadvertent injection of mutant mtDNA, but rather would represent the effects of permitted replication of a suboptimal host oocyte mtDNA complement through temporary augmentation by donated mitochondria. There could be a life-long risk of prematurely developing mitochondrial disease as age-related mtDNA mutations accumulate (Wallace, 1992; Cortopassi and Arnheim, 1993; Shigenaga et al., 1994; Brown and Wallace, 1994) on a compromised initial mitochondrial genotype. These concerns are theoretical, but the technique is so novel, and so potentially powerful, that they need to be considered. Among male children with a mitochondrial genome of suboptimal fitness,

infertility is more likely than average (Frank and Hurst, 1996), and female children might plausibly themselves undergo their own oopausal transition early (see Medvedev, 1981, for a pre-mtDNA account of the fundamentals involved: in other species there is some evidence of improvement in female-line fertility for subsequent generations if reproduction occurs at an earlier age).

Careful follow-up of the children who result from ooplasmic transfusion will be important and a detailed analysis of their mitochondrial genomes should be performed.

Conclusion: testable hypotheses

The evolutionary need for faithful inheritance of mtDNA, an asexually reproducing genome, is proposed to be the imperative behind well-known physiological and pathological phenomena in mammalian reproduction, particularly oogenesis, folliculogenesis, apparent competition between follicles, and female age-related sterility well before menopause.

Other authors have observed that mtDNA copy numbers in secondary oocytes could be only slightly greater than the mitochondrial organelle number and that mtDNA replication in secondary oocytes that are fertilized is suspended for a significant time into embryonic development. These observations require further testing, but if they are essentially correct then it is reasonable to postulate: (i) that the female germ-line mitochondrial genome is persistently haploid: that is, that during all stages of embryogenesis and oogenesis the effective number of mtDNA molecules in female germ line cells will approximate the number of mitochondrial organelles, and (ii) that the greatest restriction in mtDNA copy number between generations ('the bottleneck') will occur between the time of resumption of mtDNA replication in the embryo and the commencement of migration of the primitive germ cell from the yolk sac. It has also been postulated (iii) that competition between ovarian follicles ultimately reflects the relative mitochondrial genomic integrity of the contained oocyte; and (iv) that age-related embryonic failure (including miscarriage and sterility well before menopause in humans – the oopause) could be a consequence of a numerical shortage of qualitatively adequate mtDNA molecules among ovulated oocytes.

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