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Germline energetics, aging and female infertility

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SUMMARY

The role of metabolism in ovarian aging is poorly described, despite the fact that ovaries fail earlier than most other organs. Growing interest in ovarian function is being driven by recent evidence that mammalian females routinely generate new oocytes during adult life through the activity of germline stem cells. In this perspective, we overview the female reproductive system as a powerful and clinically relevant model to understand links between aging and metabolism, and we discuss new concepts for how oocytes and their precursor cells might be altered metabolically to sustain or increase ovarian function and fertility in women.

INTRODUCTION

Early in life's history, a complex signaling network evolved to maximize the number of descendants a cell could produce in a particular environment. This ancient network, which still exists in cells today, promotes growth and reproduction when the environment is favorable and suppresses these activities during harsh times (Kirkwood, 1987). This system explains in large part why many species gain health benefits from dietary restriction (DR) and how the body adapts to changing supplies and demands for energy. As we learn more about this survival network, it is becoming increasingly plausible to stimulate it pharmacologically. Indeed, molecules that mimic DR are in development for treatment of many aging-related health issues, such as type II diabetes, inflammation and muscle degeneration (Blum et al., 2011; Chiba et al., 2010). Despite rapid progress in this area, one aspect of human health that has been largely neglected is reproductive potential.

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DISCLOSURES

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THE OVARY AS A MODEL FOR AGING STUDIES

The main functional unit of mammalian ovaries is a multi-cellular structure referred to as the follicle (Gougeon, 1996). Each follicle is composed of an oocyte, which is a partially differentiated female germ cell arrested in prophase of the first meiotic cell division, enclosed by one or more layers of specialized somatic cells that support the oocyte during its growth. Starting with a resting (primordial) follicle that contains an oocyte and just a single layer of somatic granulosa cells, each follicle attempts to complete progressive developmental stages associated with extensive replication of the granulosa cell population and the acquirement of a second somatic cell type known as theca-interstitial cells. Through complex cell-to-cell interactions, the oocyte gains developmental competence so that it can initiate embryogenesis if fertilized after ovulation (Matzuk et al., 2002; Orisaka et al., 2009). Simultaneously, the follicular somatic cells become highly responsive to circulating factors and secrete a spectrum of hormones that exert effects both locally in the ovaries and in many other tissues including the brain, bones, skin and cardiovascular system (Buckler, 2005; Prior, 1998).

Because of the central importance of follicles to maintaining endocrine function of the female gonads as well as to fertility, ovarian lifespan is dictated by the number of follicles present in the tissue—an endpoint often referred to as the ‘ovarian reserve’. Since the 1950s, it was widely believed that females of most mammalian species are provided with a non-renewable ovarian reserve around the time of birth (Zuckerman, 1951). Following growth activation, each primordial follicle in this reserve either completes maturation for release of its enclosed oocyte at ovulation, or undergoes a degenerative process referred to as atresia. Historical studies of mouse, rat and human ovaries have shown that atresia actually claims the vast majority of follicles present in the gonads, ultimately leading to complete exhaustion of the ovarian reserve long before death due to advanced chronological age (Faddy et al., 1992; Gosden et al., 1983; Richardson et al., 1987). More contemporary work has revealed that this massive oocyte loss occurs largely through apoptosis, involving an array of genes and signaling pathways that share many similarities to the regulation of apoptosis in other organ systems (Tilly, 2001). Follicle loss can be dramatically accelerated by external insults, including chemotherapy, radiation and environmental toxicants (Tilly, 2001), leading to the premature onset of many health problems associated with natural menopause.

The concept of irreversible exhaustion of the ovarian reserve in mammals is based on the presumed absence of replicative germ cells in postnatal ovarian tissue that could give rise to new oocytes. This contrasts sharply with observations from females of non-mammalian species, including files and fish, which retain germline stem cells (GSCs) that actively support oocyte renewal during adult life (Kirilly and Xie, 2007; Nakamura et al., 2010). However, in 2004 a study was published offering multiple lines of evidence for the existence of female GSCs (or, more appropriately, oogonial stem cells or OSCs, to be consistent with the nomenclature applied to spermatogonial stem cells—their male counterparts in the adult testis) in postnatal ovaries of mice that generate oocytes to form new follicles (Johnson et al., 2004). These findings countered dogma and thus were met with skepticism by many scientists (Powell, 2007).

Nonetheless, the possibility that the ovarian reserve could be replenished became a focal point of investigation for many laboratories (Tilly et al., 2009). These efforts ultimately led to the isolation of OSCs from neonatal and adult mouse ovaries by at least three groups using different strategies (Pacchiarotti et al., 2010; White et al., 2012; Zou et al., 2009), and the purification of a similar population of oocyte-producing progenitor germ cells from adult human ovaries (White et al., 2012). In addition, studies in mice have shown that when OSCs

are reintroduced into adult ovaries, the cells differentiate to form follicle-enclosed oocytes that mature, ovulate and fertilize to produce viable embryos and offspring (White et al., 2012; Zou et al., 2009). In lower organisms, function of these types of germ cells has been tied to nutrient availability (McLeod et al., 2010; Jasper and Jones, 2010) and can even govern the pace of aging (Hsin and Kenyon, 1999; Flatt et al., 2008). These paradigm-shifting studies therefore provide a framework for introduction of OSCs into discussions of how female fertility and ovarian lifespan might be modulated in mammals.

DIETARY RESTRICTION, LONGEVITY GENES AND FEMALE FERTILITY

Mice and rats maintained on DR have reduced fertility or are completely infertile (Selesniemi et al., 2008; Visscher et al., 1952). Similarly, women below ideal body weight due to self-imposed DR have reduced fertility (Bates, 1985) and exhibit marked changes in gonadotropic hormones to levels that resemble those in women with ovarian insufficiency. Although the common wisdom is that DR negatively impacts fertility, it is less well known that DR can also have a positive impact. Almost a century ago, studies of rats noted that the "menopause has been postponed [by DR] long beyond the age at which it naturally appears" (Osborne et al., 1917). While rodents do not undergo a true menopause, this work, and several rodent studies that followed, clearly established that moderate DR extends functional ovarian lifespan in mammals.

At first glance, this finding appears at odds with the 'Disposable Soma Theory', in which the longevity of a species is a direct result of how it divides its resources between reproduction and protecting the soma (Kirkwood and Holliday, 1979). But it is not. Through analysis of physiological and ecological data on mouse survival and fertility, as well as life-history modeling, the temporary cessation of active fertility exhibited during DR in mice is believed to free up energy that can be used to enhance maintenance, thereby preserving viability and fertility for when the period of famine has passed (Shanley and Kirkwood, 2000).

Consistent with this idea, longevity can be achieved without sacrificing fertile potential in many species, including *Podospora anserina* (van Diepeningen et al., 2010), *Saccharomyces cerevisiae* (Jiang et al., 2000), *Caenorhabditis elegans* (Wood et al., 2004) and *Drosophila melanogaster* (Grandison et al., 2009). In nematodes, starvation shuts down reproduction through enforced quiescence of GSCs, which resume active gametogenesis for offspring production upon re-feeding (Angelo and Van Gilst, 2009). In flies, altering the balance of specific amino acids can increase longevity without reducing fertility (Grandison et al., 2009). The DR mimetic and SIRT1 activator resveratrol, which boosts mitochondrial function and extends lifespan in *C. elegans* and *Drosophila*, increases the number of eggs laid per organism (Wood et al., 2004). Together these data indicate that during adversity, many organisms down-regulate fertility but simultaneously up-regulate defense systems to preserve the germline. When conditions improve, fertility rebounds.

A similar situation may hold true for mammals. A stepwise 40% reduction in caloric intake in mice instituted after sexual maturation showed that while reproductive capacity is impaired during DR, these animals remain fertile much longer than continuously ad-libitum (AL)-fed controls once the DR females are allowed to resume AL feeding (Selesniemi et al., 2008). Furthermore, at advanced ages when offspring number per litter delivered by AL-fed females is close to zero, the number of pups born per litter by female mice maintained on DR for several months and then returned to an AL diet remains remarkably high (Selesniemi et al., 2008). The conclusion from these rodent studies is that if the DR regimen is mild, or if normal food intake is restored after a period of moderate DR, fertility is not negatively impacted and can actually be maintained for longer. In accordance with this, infertile women on DR rapidly regain their fertility if they increase their food consumption (Bates, 1985). An

intriguing possibility is that DR mimetics provided to women on a normal diet could provide a means to activate germ cell defense networks, thereby maintaining or restoring oocyte quality and extending fertile lifespan.

Such findings underscore the need to better define molecular mechanisms underlying organismal responses to DR or DR mimetics, and to then test these findings in the context of female fertility. The original idea that DR works *passively* by simply reducing metabolic rate or the generation of reactive oxygen species (ROS) has been largely discarded. In its place is a fundamentally different model in which DR works by triggering an *active* response that evolved to promote organismal survival during harsh conditions (Guarente, 2008; Kirkwood, 2005). At the center of this response are so-called 'longevity regulatory pathways' (Figure 1).

Although there are hundreds of longevity genes in dozens of species, four signaling pathways stand out as particularly important mediators of the DR response. These are insulin/insulin-like growth factor-1 (IGF-1) signaling, the mammalian target of rapamycin (mTOR) pathway, AMP-activated kinase (AMPK) and sirtuins. With the exception of sirtuins, which were first identified as mediators of gene silencing (Klar et al., 1979), these pathways were discovered for their roles in nutrient sensing decades before any role in aging was suspected. In the past few years, however, the fields of metabolism and aging have been united by the realization that all four of these metabolic sensors in eukaryotes form a complex regulatory network that responds to changes in a cell's internal and external environment by storing or utilizing energy for processes that boost cellular defenses against tissue damage, deterioration and disease (Canto and Auwerx, 2009; Katewa and Kapahi, 2011).

As one might expect, at least some of these pathways appear operational in female germ cells, although much of this work has focused on oocyte growth activation and not egg quality (Reddy et al., 2008; Li et al., 2010). Exceptions include reports linking mTOR and the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN) pathway, which ties into mTOR signaling, to meiotic progression in oocytes and embryonic genome activation in fertilized eggs, respectively (Lee et al., 2012; Zheng et al., 2010). In addition, several sirtuin family members are expressed in mouse oocytes. Of particular interest, loss of sirtuin-3 function in mouse eggs increases mitochondrial ROS production, leading to impaired pre-implantation embryonic development after fertilization (Kawamura et al., 2010).

EFFECTS OF DIET ON EGG QUALITY

Although early reports indicated that reduced dietary food intake can slow follicle depletion from ovaries in rats and mice (Lintern-Moore and Everitt, 1978; Nelson et al., 1985), the ovarian reserve in aged females subjected to DR followed by AL feeding is, like their age-matched AL-fed counterparts, severely diminished when compared to that of young adult females (Selesniemi et al., 2008). Thus, the beneficial effects of DR on female fertility, fecundity and offspring survival are apparently not due to maintenance of a larger follicle reserve with age. While it is conceivable that the benefits of DR in this model are partly a result of improved capacity of the uterus of aged females to establish and support a pregnancy, oocyte donation studies in humans have demonstrated that aging-related infertility can be effectively overcome by use of oocytes from young adult donors (Klein and Sauer, 2002; Sauer et al., 1992). In fact, given that women in their sixties have successfully carried pregnancies to term as surrogates (Paulson et al., 2002; Sauer et al., 1995), the single most important factor for determining pregnancy success rates in women

of advanced maternal age seems to be oocyte quality and not uterine dysfunction (Navot et al., 1991).

Production of a developmentally competent egg requires the successful completion of meiosis, ultimately reducing its chromosome number to one-half once penetrated by the sperm at fertilization. The joining of the male and female pronuclei in the fertilized egg then restores a normal chromosome complement in the newly formed embryo. Unfortunately, the meiotic cell cycle is highly prone to errors with increasing age, leading to aneuploid oocytes (Hassold and Chiu, 1985; Hassold and Hunt, 2009; Hunt, 1998). Even with ovulation continuing in women into their early forties, the quality of oocytes ovulated by women as they grow older becomes compromised, elevating the risk for fertilization or embryonic failure, miscarriage and birth defects. The most widely known example of this maternal aging effect is the dramatic rise in risk for conception of offspring with trisomy 21 or Down syndrome, which increases from around 2% of clinical pregnancies for women in their twenties to 30% or more of clinical pregnancies for women in their forties (Hassold and Chiu, 1985).

The clinical importance of overcoming this aging-related decline in oocyte quality has become much more relevant as increasing numbers of women bear children in the second half of their fertile period (Matthews and Hamilton, 2009; Ventura, 1989). Compounding this problem is the inherent difficulty in circumventing fertility issues even with assisted reproductive technologies such as in-vitro fertilization (IVF). Since several factors contribute to the decline in oocyte quality with advancing age, a widely held assumption in the field of human reproduction is that any single or simple pharmacological intervention will be insufficient to overcome this problem. However, very recent studies with mice indicate that this fundamental belief, like that of a fixed ovarian reserve at birth, may be invalid. For example, recent studies show that female mice maintained on DR for 7 months and then allowed to AL feed for one month do not exhibit any of the hallmark features of deteriorating egg quality with age observed in AL-fed control females (Selesniemi et al., 2011). Notably, the increased incidence of aneuploidy, meiotic spindle abnormalities, chromosomal misalignment, mitochondrial aggregation and declining ATP levels detected in oocytes of aged AL-fed females are all absent in oocytes of aged females previously maintained on DR. Although additional studies are needed to address the mechanisms underlying these striking beneficial effects, aging-related aneuploidy and spindle defects in eggs at least no longer appear to be unreachable targets for therapeutic manipulation.

ENERGY, AGING AND THE ROLE OF MITOCHONDRIA

Whether one is working on metabolism or aging, it is hard to ignore mitochondria, the structures at the center of cellular energy production and utilization. These organelles are essential for generating most of the ATP in the body, which in humans amounts to about 65 kg per day to meet basic metabolic demands (Tornroth-Horsefield and Neutze, 2008). Other key functions of mitochondria include calcium buffering, reduction-oxidation (redox) homeostasis and programmed cell death (apoptosis). Mitochondria are continuously moving throughout the cell—undergoing fusion, fission, and degradation—to eliminate and replace damaged organelles, and to meet fluctuating energy needs (Palmer et al., 2011).

Data from flies, rats, mice, monkeys and humans show that as tissues age, both the number and activity of mitochondria decline, compensated by an increase in their overall size (Cho et al., 2011; Ferguson et al., 2005; Short et al., 2005; Wallace, 2001). Mitochondrial dysfunction is associated with, and potentially contributes to, common aging-related diseases such as atherosclerosis, obesity-induced type II diabetes, sarcopenia and neurodegenerative disorders (Di Lisa et al., 2009; Lin and Beal, 2006; Wallace, 2001).

Underlying processes include a decline in mitochondrial membrane potential and ATP output, increased activation of the mitochondrial permeability transition pore (mPTP), mitochondrial membrane depolarization and leakage of mitochondrial matrix solutes into the cytoplasm (Di Lisa et al., 2001; Hafner et al., 2010; Liu et al., 2011; Wallace, 2001).

In addition to aging and aging-related diseases, considerable evidence supports a role for mitochondria as mediators of the benefits of DR in rodents and humans (Cerqueira et al., 2011; Civitarese et al., 2007; Lopez-Lluch et al., 2006; Lopez-Lluch et al., 2008). In a variety of species (Guarente, 2008; Johannsen and Ravussin, 2009), DR increases mitochondrial number and function. A recent rat study, however, did not observe this change (Hancock et al., 2011). The sirtuin-1 (SIRT1)-AMPK network, considered a mediator of DR physiology, acts to raise both the number and activity of mitochondria (Gerhart-Hines et al., 2007), as do DR mimetics that stimulate SIRT1 or AMPK activity, such as resveratrol (Baur et al., 2006; Feige et al., 2008; Funk et al., 2010; Lagouge et al., 2006), SRT1720 (Minor et al., 2011) and metformin (Canto et al., 2009; Suwa et al., 2006). In flies and nematodes, changes in mitochondrial metabolism are known to be necessary (Bahadorani et al., 2010; Bishop and Guarente, 2007; Zid et al., 2009) and sufficient (Bahadorani et al., 2010; Durieux et al., 2011; Rera et al., 2011) for DR to extend lifespan.

MITOCHONDRIA AND OOCYTE COMPETENCY

Of the many potential mechanisms by which DR benefits oocytes, one of the more plausible is prevention of abnormal mitochondrial aggregation and decreased ATP levels that occur in oocytes of aged females (Selesniemi et al., 2011). Many studies have proposed a link between insufficient ATP availability in eggs and defective chromosomal segregation—an outcome that probably ties to meiotic spindle abnormalities (Eichenlaub-Ritter et al., 2004; Schon et al., 2000; Zheng et al., 2007). Defective spindle formation would result in a reduced capacity for successful fertilization and the failure of zygotes produced from energetically compromised eggs to form viable blastocysts (Bentov et al., 2010). Consistent with this idea, disruption of mitochondrial oxidative phosphorylation in mouse oocytes results in reduced potential for meiotic maturation and fertilization, as well as decreased pre-implantation embryonic developmental potential (Van Blerkom et al., 1995).

Other studies with mice have demonstrated that failure of oocytes to adequately readjust ATP levels after sperm penetration disrupts intracellular calcium oscillations (Igarashi et al., 1997; Igarashi et al., 2005), which are critical for immediate post-fertilization events that ensure developmental competency of the embryo (Dumollard et al., 2004; Vitullo and Ozil, 1992). In human eggs, higher ATP levels have been correlated with a greater potential for successful embryonic development and implantation (Van Blerkom et al., 1995). These findings, along with observations that mitochondria in oocytes of women in their forties frequently exhibit swelling and abnormal cristae (Muller-Hocker et al., 1996), collectively support the idea that impaired bioenergetic capacity in oocytes is a primary contributor to declining egg and embryo quality with advancing maternal age.

Additional aspects of mitochondrial physiology must be considered when evaluating a central role for these organelles in oocyte development, meiotic maturation, fertilization and pre-implantation embryonic competency. Obviously, the generation of ROS during the oxidative phosphorylation steps associated with ATP production is closely linked to mitochondrial bioenergetics. In turn, it may be of little surprise that studies of mice have reported chronic antioxidant treatment throughout adult life can sustain egg quality in aging females (Tarin et al., 2002a). Unfortunately, the function of other cells and tissues in the reproductive tract is simultaneously impaired by this approach, with excessive fetal loss and diminished offspring numbers noted (Tarin et al., 2002b). Accordingly, long-term systemic

administration of antioxidants has little, if any, clinical application for improving human female fertility.

On a more basic level, a striking aspect of mitochondrial physiology that warrants consideration is the amplification of mitochondrial numbers as human oocytes develop from their most immature state containing between 5–10 X 10^3 mitochondria, to mature metaphase II eggs containing 1–5 X 10^5 or more mitochondria (Jansen and Burton, 2004; Piko and Matsumoto, 1976). The reasons for this tremendous expansion of mitochondrial numbers during oocyte maturation are not fully understood. One belief is that the oocyte is actively preparing itself for the increased energy demands of successful fertilization and early cleavage divisions associated with embryonic development. This seems reasonable, especially if one considers that the machinery required for mitochondrial replication is shut off at the metaphase II egg stage and is not reactivated in the embryo until after the blastocyst implants in the uterine wall (Larrson et al., 1998). Accordingly, one would expect a steep decline in mitochondrial numbers as an embryo expands from the one-cell zygote to a blastocyst containing a hundred or more cells, a prediction supported by assessment of mitochondrial DNA (mtDNA) content during pre-implantation embryogenesis (Spikings et al., 2007; Wai et al., 2008) and mitochondrial numbers per cell in blastocyst-stage embryos (Van Blerkom, 2008).

It bears mentioning that mtDNA content rather than absolute numbers of mitochondria, which contain 1–10 copies of mtDNA per organelle, may actually be a more reliable indicator of the competence of a given oocyte. Past studies have shown that mtDNA copy numbers in oocytes and early-stage embryos positively correlate with fertilization and developmental potential, respectively (Almeida-Santos et al., 2006; Spikings et al., 2006). In fact, successful embryogenesis has been tied to threshold levels of mtDNA content per egg at the time of fertilization, with those oocytes at the low range of this threshold more prone to failed maturation, reduced fertilization rates and embryonic developmental arrest (El Shourbagy et al., 2006; Piko and Taylor, 1987; Reynier et al., 2001; Santos et al., 2006; Wai et al., 2010). In addition, studies of porcine oocytes have revealed that suppression of mtDNA replication during in vitro maturation to the metaphase II egg stage results in reduced fertilization competence as well as pre-implantation embryonic developmental arrest, and that the severity of these outcomes is tightly linked to a minimal threshold copy number of mtDNA (Spikings et al., 2007).

Another intriguing feature of the oocyte is that its mitochondria tend to be very small (≤1 μm in diameter), with electron-dense matrices and few cristae. Despite these structural features, oocyte mitochondria are highly active and produce the majority of energy needed by the egg and early embryo (Dumollard et al., 2007; Motta et al., 2000; Van Blerkom et al., 1995). After fertilization, mitochondria in developing embryos undergo striking ultrastructural changes. By the time of blastocyst formation, these organelles have acquired an elongated appearance with complex cristae and less electron-dense matrices, more typical of mitochondria in somatic cells (Sathananthan and Trounson, 2000; Van Blerkom, 1989a, 1989b, 1993; Van Blerkom and Motta, 1979; Van Blerkom et al., 1973). The significance of this mitochondrial remodeling is unclear; however, human embryos that arrest in vitro often contain mitochondria that have failed to transform into a more orthodox morphology, a step that is presumably necessary to meet the energy demands of the developing embryo (Van Blerkom, 1989a).

Finally, mitochondria in oocytes play another critical role in reproduction: they serve as the source for uniparental inheritance of the mitochondrial genome from one generation to the next. It is fairly well established that paternal (sperm-derived) mitochondria are degraded in newly formed embryos within the first few cleavage divisions, leaving maternally derived

mitochondria as the sole pool for replication of these organelles in the embryo and resultant offspring (Cummins, 1998; Giles et al., 1980; Hutchinson et al., 1974; Kaneda et al., 1995; Sutovsky et al., 2000). The mechanisms underlying this sex-specific selection against the transmission of paternal mtDNA are not completely understood. In many species, however, the selection appears to result from ubiquitination of sperm-derived mitochondria, which allows for their subsequent removal from the embryo (Sutovsky et al., 2004). Equally unclear is why paternal mtDNA inheritance is actively selected against. One idea is that it minimizes the transmission of mtDNA mutations that arise in sperm exposed to ROS during spermatogenesis (Aitken, 1995). Consistent with this idea, sperm mitochondria often harbor mtDNA mutations and deletions (Reynier et al., 1998), which in turn are linked to poor sperm motility and malefactor infertility (Kao et al., 1995; Ruiz-Pesini et al., 2000; St. John et al., 2001).

Irrespective of the mechanisms that drive uniparental mtDNA inheritance or the advantages it provides, this process necessitates strict maintenance of mtDNA integrity in the female germline. Otherwise, a cumulative mutational disaster could occur, as mitochondria from the oocyte are used to seed the new embryo in each successive generation (Jansen and De Boer, 1998). To ensure asexual maintenance of mitochondrial genome integrity, a multistep process has been proposed that involves an initial quantitative restriction on mtDNA genotypes that will potentially be passed (the so-called ‘mitochondrial bottleneck’), followed by a period of tremendous amplification. Then, under pressure to achieve improved fitness in subsequent generations, a competitive mass selection occurs (Jansen, 2000). The quantitative restriction event takes place in the developing embryo as mtDNA content in the embryo, and mitochondrial numbers per cell, decline exponentially from peak levels at the fertilized egg stage to extremely low levels in the implanting blastocyst. In fact, embryonic primordial germ cells contain 10 or fewer mitochondria per cell, in stark contrast to the hundreds of thousands of mitochondria present in each egg (Jansen and Burton, 2004; Piko and Matsumato 1976). This latter point exemplifies the importance of the second step in the process of mtDNA selection, which entails a period of tremendous amplification with minimal selection that presumably allows for some degree of genetic drift (Brown et al., 2001). The third and final stage of the process—competitive mass selection—is the least established in terms of actual mechanism.

One theory is that constant culling of oocyte-containing follicles by atresia serves to identify a given follicle that will release an egg at ovulation with the highest degree of mtDNA integrity (Jansen and Burton, 2004; Jansen and De Boer, 1998). Although this is an attractive proposal, direct evidence for it is currently lacking. However, the existence of a tightly controlled surveillance system for ensuring maternal mtDNA integrity is supported by observations that mtDNA deletions are usually not transmitted to the offspring of clinically symptomatic women. In addition, the common $\Delta mtDNA^{4977}$ deletion is greater in unfertilized human eggs than in early cleavage-stage embryos (Brenner et al., 1998; Perez et al., 2000), and two-thirds of degenerated or arrested oocytes carry the $\Delta mtDNA^{4977}$ deletion (Duran et al., 2011).

ENERGETICS, AGING AND FEMALE FERTILITY: CONNECTING THE DOTS

Direct causative relationships between impaired mitochondrial function, suboptimal bioenergetic capacity and reduced developmental competency of eggs in aging females have not yet been unequivocally established. Nevertheless, evidence for such relationships continues to solidify, as does the link between longevity pathways and fertility. Studies of hamsters and mice have reported that maternal aging is associated with significant decreases in ATP content and mitochondrial numbers in oocytes. In addition, the demonstration that DR instituted during adulthood not only benefits multiple parameters of egg quality in aging

female mice (Selesniemi et al., 2011), but also extends the natural reproductive period (Selesniemi et al., 2008), argues strongly that the same signaling pathways mediating responses of somatic cells to DR are also at work in the female germline. Further supporting this conclusion are recent data showing that sirtuins are expressed in rat and mouse oocytes (Kawamura et al., 2010; Luo et al., 2012), that DR increases the expression of sirtuins in the rat ovary (Luo et al., 2012), and that the development of embryos arising from oocytes lacking mitochondrial-associated sirtuin-3 is significantly impaired (Kawamura et al., 2010).

Although progress has been made in connecting the dots, we are still far from understanding or manipulating the bioenergetic and longevity pathways that impact female fertility. Thus, the experimental and clinical data currently in hand can be viewed as opening chapters in a saga that may one day offer unprecedented opportunities for the clinical management of egg quality, fertilization and pre-implantation embryogenesis in human assisted reproduction. Looking ahead, we will conclude with two examples of potential future chapters that integrate many of the concepts discussed herein.

AUTOLOGOUS GERMLINE MITOCHONDRIAL ENERGY TRANSFER AND EGG QUALITY

In the mid-to-late 1990s, 27 female subjects who had repeatedly failed to become pregnant following assisted reproduction due to poor embryo quality and implantation failure participated in a trial of a new fertility protocol termed ooplasmic transfer (Barritt et al., 2001; Brenner et al., 2000; Cohen et al., 1997; Cohen et al., 1998; Harvey et al., 2007). Under the assumption that the recurrent failure of these women to achieve pregnancies was due, at least in part, to an age-related impairment in the quality of their eggs, their next cycle of IVF included transfer of a small amount of cytoplasm extracted from young donor oocytes (viz., obtained from different women) into their oocytes. Thirty attempts of ooplasmic transfer were performed by the first clinic, resulting in 13 live births (17 babies total, comprised of 11 singletons, 1 set of twins, and 1 set of quadruplets) and 1 first-trimester miscarriage (45, XO karyotype). The twin pregnancy resulted in birth of a female with a normal karyotype (46, XX) and a chromosomally abnormal sibling (45, XO). Of the babies born, the rate of chromosomal abnormalities (1 of 17, or 5.9%) was within the normal range of IVF outcomes for women at the ages tested in that region of the United States (Harvey et al., 2007). The high pregnancy success rates achieved in this relatively small cohort of patients, who had repeatedly failed all prior IVF attempts, raised hopes that human assisted reproduction would finally have a new tool in its arsenal to combat infertility associated with poor egg and embryo quality.

Other clinical sites were quick to join in (Lanzendorf et al., 1999); however, enthusiasm for widespread adoption of ooplasmic transfer as a clinical protocol was short-lived, in part because of concerns about mitochondrial heteroplasmy (Barritt et al., 2001; Brenner et al., 2000). Although the procedure involved transfer of cytoplasm from donor eggs, and not purified mitochondria, it is widely believed that the benefit to recipient eggs came from the transfer of active donor mitochondria (Bentov et al., 2011; Van Blerkom et al. 1998). This conclusion has been substantiated by animal studies (El Shourbagy et al., 2006; Yi et al., 2007) and, to a degree, by follow-up studies of the children born through ooplasmic transfer who carry mitochondria from both the biological mother and the egg donor (Brenner et al., 2000; Barritt et al., 2001). At present, the negative health impact, if any, of heteroplasmy in these children is unknown, but animal models indicate there may be at least some cause for concern. For example, studies in mice show that mitochondrial heteroplasmy can produce an adult-onset phenotype consistent with metabolic syndrome (Acton et al., 2007). Other work has shown that heteroplasmy can also negatively impact cognitive function (Sharpley et al., 2012).

Additionally, oocyte mitochondria contain genetic material that is distinct from nuclear genes contributed by the biological mother and father. Accordingly, the children conceived following this procedure possess genetic material derived from not two but three distinct sources: the biological mother, the biological father and the egg donor. Aside from an array of potential ethical and legal issues associated with heterologous ooplasmic transfer, the U.S. Food and Drug Administration (FDA) viewed this procedure as genetic manipulation of human germ cells for the purpose of generating embryos. Thus, in 2001 the FDA ruled that heterologous ooplasmic transfer could no longer be used for human assisted reproduction unless the procedure was submitted for review and testing under Investigational New Drug (IND) guidelines (Zoon, 2001). While use of autologous mitochondria from a woman's own somatic cells would avoid mitochondrial heteroplasmy, somatic mitochondria are prone to aging-related mtDNA damage resulting in heritable mutations. Introduction of these mitochondria into oocytes at fertilization could lead to propagation of mutant mitochondria in newly formed embryos and resultant offspring, a risk too great to consider for clinical protocol development.

However, the discovery that OSCs are present in ovaries of not just adult mice but also of reproductive age women (Johnson et al., 2004; White et al., 2012; Zou et al., 2009) has opened prospects for bringing a modified version of ooplasmic transfer into clinical practice. Termed AUGMENT (for autologous germline mitochondrial energy transfer; Woods et al., 2013), this procedure would provide an autologous germline-derived source of cytoplasmic extract or purified mitochondria for the bioenergetic reinvigoration of eggs whose capacity for fertilization and embryogenesis has been compromised by aging (Figure 2).

The use of OSCs as a source of mitochondria for enhancing egg and embryo quality is attractive for reasons other than simply being a patient-matched source of these important energy-boosting organelles. First, since OSCs function as natural precursor cells for the generation of oocytes (White et al., 2012; Zou et al., 2009). If mitochondria in the female germline are indeed managed quite differently from those in somatic cells, the use of OSCs as a source of mitochondria for rejuvenation of eggs would be compatible with the natural processes of surveillance and selection that govern maternal mitochondrial transfer from one generation to the next. Second, OSC mitochondria, being derived from slowly dividing stem/progenitor cells, are more likely to be free of cumulative damage to their genomes than mitochondria in patient-matched somatic cells. Preliminary observations from studies of human OSCs support this contention (Woods and Tilly, 2013). Perhaps equally important are observations that the bioenergetic potential of mitochondria in human OSCs, as measured by ATP generation over time, far exceeds that of equivalent numbers of mitochondria isolated from several other human cell lineages, including embryonic and adult somatic stem cells (Woods and Tilly, 2013). Finally, the cell lineage-specific transfer of key nuclear-encoded proteins into mitochondria would be better preserved through the use of OSCs versus non-germline cells. Given all of these considerations, along with the preclinical and clinical proof-of-concept data available from prior studies discussed above, the use of AUGMENT for safely improving human assisted reproduction without the ethical, legal and biological issues surrounding heterologous ooplasmic transfer is an exciting prospect to consider.

MITOCHONDRIAL ACTIVATORS TO BOOST EGG QUALITY

Another potential approach to overcoming energy deficits in eggs is to identify new biological and chemical entities that enhance mitochondrial numbers or the efficacy of mitochondrial ATP generation in oocytes. The development of such compounds that safely reproduce the striking benefits of DR on egg quality in aging females (Selesniemi et al., 2011) could represent a significant leap forward in human assisted reproduction. The main

hurdle faced is the extreme rarity of oocytes for conducting large-scale mitochondrial screening assays, coupled with the cost and complexities of performing aging studies with mice. However, the availability of mouse and human OSCs, which can be maintained and expanded ex vivo to generate essentially unlimited numbers of cells for screening (White et al., 2012), may provide a solution (Figure 3). Because these cells function as natural oocyte progenitors, it is reasonable to predict that compounds identified as mitochondrial boosters in OSCs would exhibit similar properties in oocytes. In addition, use of OSCs as a screening platform offers an opportunity to map molecular events through which a given compound, or family of compounds, boosts mitochondrial numbers or activity in female germ cells. Such studies would further benefit by generating OSC lines that carry desired manipulations of key components of the longevity regulatory pathways discussed earlier (Figure 1). Establishment of these types of female germ cell lines could facilitate identification of control points that coordinate bioenergetic potential in oocytes. In turn, this may lead to a more directed approach for identifying potential lead compounds that could be tested in aging female mice for their ability to improve egg quality.

There is ample reason to believe that IVF outcomes for patients with energetically compromised eggs or embryos would benefit from mitochondrial activators (Bentov et al., 2010; Van Blerkom, 2011), similar to the energetic boost provided to eggs by direct mitochondrial transfer. However, mitochondrial activators may also prove valuable as in-vivo agents to ensure that oocytes ultimately released from the ovaries at ovulation or retrieved from IVF patients after ovarian stimulation are fully prepared, from a bioenergetic perspective, to undergo the final maturational steps needed for full developmental competency. One example of their potential utility would be to combat the increase in oocyte aneuploidy associated with maternal reproductive aging, a process that, at least in mice, has been tied to mitochondrial dysfunction and energy deficits in the ovulated eggs (Selesniemi et al., 2011).

Mechanistically, the formation and maintenance of the meiotic spindle is an energy-driven process that is highly susceptible to failure in oocytes of aged females (Figure 4). The resultant chromosomal misalignment or unequal chromosomal segregation produces an egg with too few or too many chromosomes, leading to genetic errors that can be passed to resultant embryos after fertilization (Gaulden, 1992). Clinically, maternal aging-related increases in egg and embryo aneuploidy are tied directly to parallel increases in trisomic conceptions, implantation failures and miscarriages (Benadiva et al., 1996; Hassold and Chiu, 1985; Munné and Cohen, 1998; Munné et al., 1995). Identification of orally active compounds that boost the energetic capacity of oocytes prior to ovulation or retrieval for IVF may therefore provide a novel strategy to maximize the chances of obtaining eggs from females at advanced reproductive ages that are free from genetic errors and other problems that contribute to post-fertilization embryonic failure. In turn, such strategies might also mitigate the maternal aging-related increase in risk for miscarriage and birth defects, including Down syndrome.

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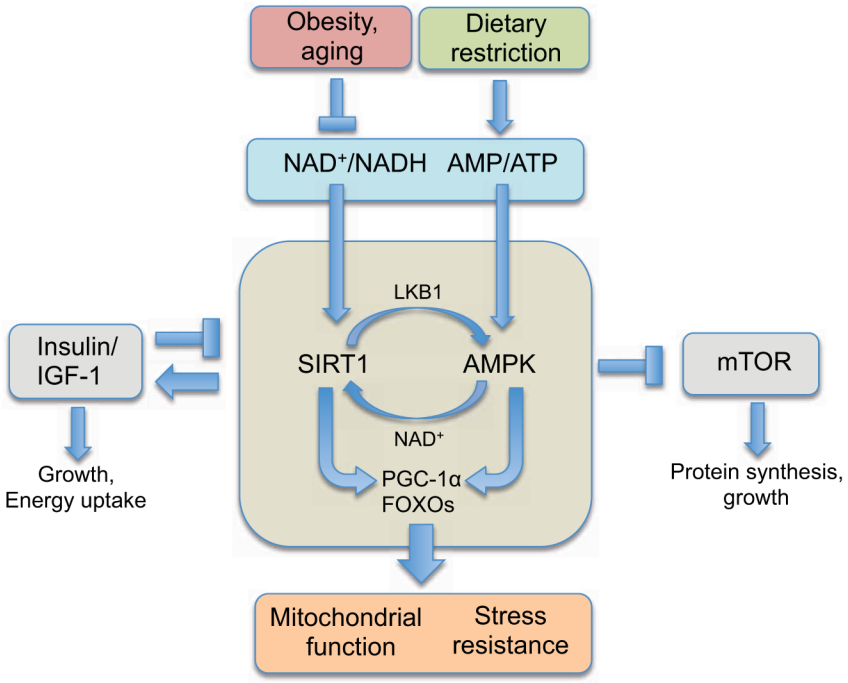

Figure 1. Longevity pathways that promote health and survival

Current data indicate that environmental signals alter the pace of aging by modulating key metabolic sensors, such as SIRT1 and AMPK. These pathways interact with both mTOR and insulin/IGF-1 to control cell growth and energy intake. Obesity and aging reduce the ratios of NAD^+/NADH and AMP/ATP, whereas DR has the opposite effect. Downstream, the actions of two transcriptional regulators, PGC-1α and FOXO, induce mitochondrial function and stress resistance, among other protective mechanisms. Together, this network coordinates cellular responses to stress, nutrient availability and metabolic demands, with mitochondria as key nexus points.

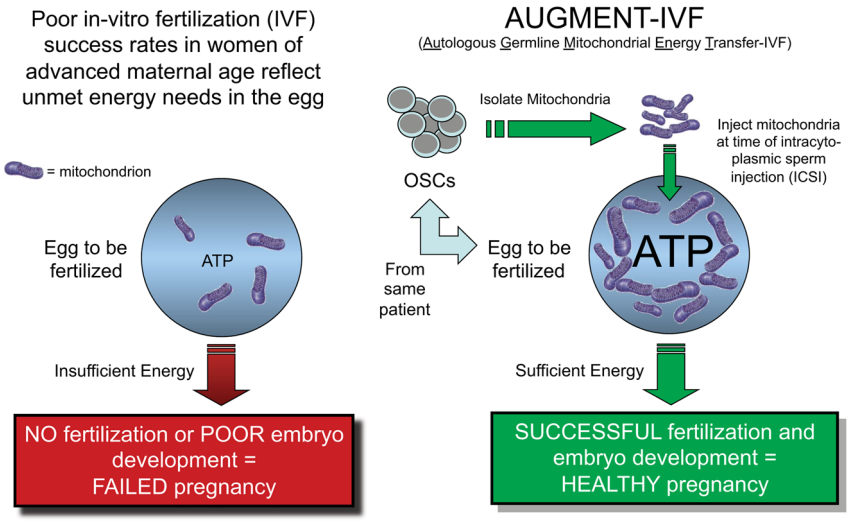

Figure 2. Proposed method of improving human IVF outcomes with AUGMENT

Reduced mitochondrial activity and bioenergetic potential contribute to aging-related impairments in female fertility, even with the use of assisted reproductive technologies such as IVF. Delivery of mitochondria derived from a patient's own natural egg precursor cells (OSCs) into that same patient's eggs during intracytoplasmic sperm injection raises the threshold level of mitochondria in that egg, providing sufficient energy to support successful fertilization and embryogenesis.

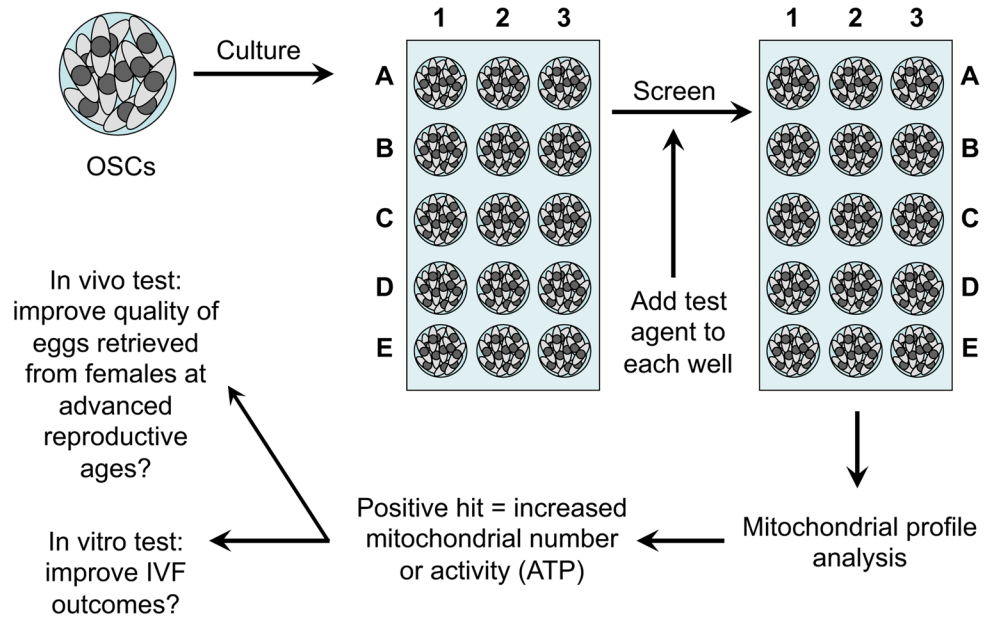

Figure 3. Identification and utility of female germ cell mitochondrial boosters

Culture of human OSCs, which are natural precursor cells for human oocytes, allows high-throughput screening of biological and pharmacological entities for their ability to increase various aspects of mitochondrial dynamics, including mtDNA content, mitochondrial membrane potential, and ATP-generating capacity. Positive hits can be further tested using a combination of in vitro and in vivo assays to assess if aging-related impairments in egg quality, embryonic developmental competence and fertility can be minimized.

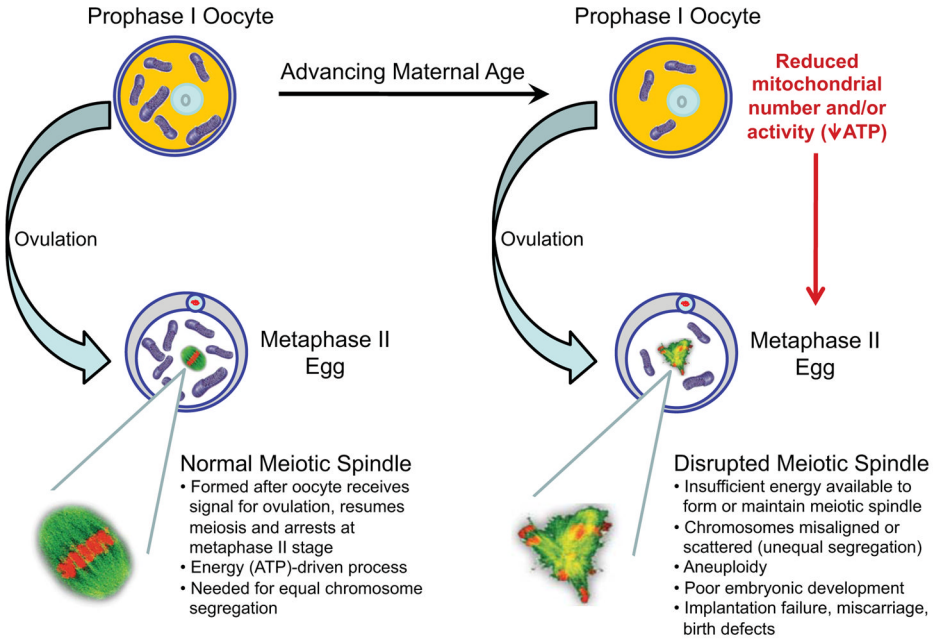

Figure 4. Negative impact of maternal aging on ovulated egg quality and female fertility

Schematic depiction of how maternal aging-associated deficits in bioenergetic potential in oocytes compromises their ability to form fertilization-competent eggs. Insufficient energy in the eggs of older females leads to impaired meiotic spindle formation and maintenance, unequal segregation of genetic material at the completion of meiosis during fertilization, and aneuploid conceptions, which result in pre-implantation embryonic growth arrest, implantation failure, miscarriage and birth defects.