Supporting Information

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SI Materials and Methods

Animals. Adult B6D2F1 male mice were obtained from The Jackson Laboratory. Virgin C57BL/6 female mice were obtained from the National Institute on Aging (Bethesda, MD) for oocyte yield/maturation and gene expression studies, or from The Jackson Laboratory for aneuploidy, spindle, and mitochondrial studies. For each experiment performed, endpoint comparisons between AL-fed and CR mice were always made using animals from the same supplier. Mice with a targeted disruption of the *Pgc-1α* gene (1) were obtained as heterozygous breeders from B. M. Spiegelman (Harvard Medical School, Boston, MA) to generate wild-type and homozygous-null female mice from the same colony for direct comparisons. All experiments were reviewed and approved by the institutional animal care and use committee of Massachusetts General Hospital.

Feeding Regimen. We used an adult-onset CR protocol developed by the National Institute on Aging in their Biomarkers of Aging Study (2), in which CR is initiated at 3.5 mo of age in a stepwise manner over a 2-wk period to achieve 40% restriction at 4 mo of age. Each female was housed individually in a conventional (nonventilated) cage and fed once daily with a rationed amount of fortified rodent diet (National Institute on Aging). The fortified rodent diet is supplemented with vitamins and minerals such that daily intake of these micronutrients is comparable to that of control animals with ad libitum (AL) access to the nonfortified (standard) rodent diet (2). Diet composition is otherwise identical. The CR protocol was continued until 11 mo of age, at which time the mice maintained previously on CR were allowed AL access to standard rodent diet for 1 mo. This procedure was followed on the basis of prior work showing that female mice maintained on CR during adulthood continue to breed and deliver offspring into advanced ages after their return to an AL diet (3). Water was provided AL for all animals during the entire study period. All animals were housed together in pathogen-free facilities, monitored continuously by sentinel animals present on each rack, for the entire study period. To ensure pathogen-free status, each sentinel cage received bedding from all other cages in that rack upon cage change every 2 wk. Blood was drawn from sentinel animals every 12 wk to test for antigens indicative of pathogen infection, and one sentinel animal per cage was killed every 36 wk for complete health assessment.

Body Weight and Reproductive Cyclicity. To confirm that the CR protocol was working as expected, the weight of each mouse was taken just before the start of the CR protocol (3 mo of age), at the conclusion of the CR protocol (11 mo of age), and 1 mo following the return of CR mice to AL feeding (12 mo of age) (Fig. S6). In addition, past studies that used alternating days of fasting and feeding to achieve CR in female mice reported that aging-related disruption of estrous cyclicity was delayed by food restriction (4). These data, along with more recent observations that adult-onset CR delays the timing of reproductive failure in female mice as tested in natural mating trials (3), support that the approach maintains cyclic production of reproductive hormones required for normal 4–5 d estrous cycles. To further confirm this under the feeding protocol used here to achieve CR, daily vaginal cytological smears were assessed, as described (5), to compare estrous cyclicity in aged AL-fed and CR–AL-fed mice over a 30-d period (Fig. S7). It is well established in mice that female reproductive aging is associated with a shift from typical 4- to 5-d estrous cycles to prolonged cycles lasting more than 5 d (7). For example, the proportion of young adult C57BL/6 mice exhibiting cycles lasting 4–5 d versus more than 5 d is ~80 and 20%, respectively; however, by 12 mo of age nearly two-thirds of female mice exhibit prolonged estrous cycles indicative of pending ovarian failure (6, 7) (Fig. S7).

Oocyte Retrieval and Classification. Young adult (3-mo old) and aged adult (12-mo old) AL-fed and CR–AL-fed female mice were superovulated with an i.p. injection of pregnant mare serum gonadotropin (PMSG) (10 IU; Sigma-Aldrich) followed by human chorionic gonadotrophin (hCG) (10 IU; Sigma-Aldrich) 46–48 h later. Oocytes were collected 15–16 h after hCG injection by puncturing the oviducts with an insulin syringe. Retrieved oocytes were denuded of cumulus cells by a brief incubation in 80 IU/mL of hyaluronidase (Sigma-Aldrich), followed by three washes with human tubal fluid medium (HTF) (Irvine Scientific) supplemented with 0.4% BSA (fraction V, fatty acid free; Sigma-Aldrich). Oocytes were counted and classified using a Hoffman light microscope as mature metaphase II (MII; presence of first polar body in the perivitelline space), maturation arrested (germinal vesicle breakdown with no polar body extrusion or germinal vesicle intact) or dead (condensed, fragmented cytoplasm). Oocytes from the three experimental groups of females were always analyzed in parallel.

In Vitro Fertilization (IVF) and Embryo Culture. The cauda epididymides and vas deferens were removed from adult B6D2F1 male mice and placed into HTF medium supplemented with BSA. Sperm were obtained by gently squeezing the tissue with tweezers and then capacitated for 30 min at 37 °C. Denuded MII oocytes or cumulus cell–oocyte complexes were mixed with $1–2 \times 10^6$ sperm/mL in HTF medium supplemented with BSA for 6–9 h. Inseminated oocytes were then washed of sperm and transferred to fresh medium. The number of two-cell embryos formed between 24 and 48 h postinsemination was used as a measure of IVF success rate (8). At 48 h postinsemination, oocytes and embryos were then transferred to 50-μL drops of potassium simplex optimized media (KSOM) (Irvine Scientific) supplemented with 10% FBS, and the drops were covered with mineral oil to support preimplantation embryonic development. Light microscopic examination was then performed every 24 h for a total of 120 h to monitor blastocyst development rates (8). Oocytes from the three experimental groups were always analyzed in parallel, and all cultures were maintained in the same humidified incubator at 37 °C under 5% CO_2 in air.

Chromosomal Analysis. A total of 795 mature (MII) oocytes collected from 3-mo-old AL-fed (n = 20 mice), 12-mo-old AL-fed (n = 34 mice), and 12-mo-old CR–AL-fed (n = 20 mice) females were fixed individually for chromosomal analysis using Tarkowski's method (9), as described (10). All preparations were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) and scored for aneuploidy rates using a fluorescence microscope. The criteria used for assessing aneuploidy were to score an oocyte with more than 20 chromosomes as hyperploid and an oocyte containing at least 13 but less than 20 chromosomes as hypoploid. Oocytes that possessed clearly separated sister chromatids were scored as exhibiting premature sister chromatid separation (PSCS). Oocytes with excessive chromosomal clumping or spreading were excluded from analysis. To minimize the possibility of treatment effects being ascribed to

an artifactual loss of chromosomes or sister chromatid separation during sample preparation, oocytes from the three groups were always analyzed in parallel, and a single trained observer performed all preparations and scoring in a blinded fashion.

Immunofluorescence. Mature oocytes were collected by superovulation, washed in PBS containing 0.5% BSA, and briefly incubated in acidified Tyrode's solution (Irvine Scientific) to soften and remove the zona pellucida. Afterward, the oocytes were extensively washed and then fixed in 2.0% neutral-buffered paraformaldehyde containing 0.5% BSA. Permeabilization and blocking was performed by incubating the oocytes in mouse blocking solution (Vector Laboratories) supplemented with 0.5% BSA, 0.1% Triton X-100, 0.05% Tween-20, and 5% normal goat serum. Oocytes were then washed and incubated overnight in a 1:200 dilution of mouse anti–α-tubulin antibody (Sigma-Aldrich) in PBS containing 0.5% BSA, washed and subsequently incubated with a 1:250 dilution of goat antimouse IgG conjugated with Alexa Fluor-488 (Life Technologies). After washing, oocytes were mounted using Vectashield containing propidium iodide (Vector Laboratories) and analyzed by confocal microscopy in a blinded fashion. For the spindle analyses, oocytes with barrel-shaped bipolar spindles having distinct and well-organized microtubule fibers, along with tightly aligned chromosomes on the metaphase plate, were scored as normal.

Mitochondrial Analysis. Mature oocytes were collected by superovulation, denuded of adherent somatic (cumulus) cells, and incubated in HTF medium supplemented with 0.4% BSA and 200 nm MitoTracker Red CMRox (Life Technologies) for 60 min at 37 °C. Oocytes were then washed and incubated in acidified Tyrode's solution, washed, fixed, and washed again. Oocytes were then incubated in PBS containing 0.5% BSA, 0.05% Tween-20, and 0.1% Triton X-100 for 1 h, mounted using Vectashield, and analyzed by confocal microscopy in a blinded fashion. Oocytes with a uniform cytoplasmic distribution of active mitochondria were scored as normal. Levels of ATP in individual mature oocytes from the three groups of mice were determined using a commercially available bioluminescent assay kit (Sigma-Aldrich).

Gene Expression. Levels of *Pgc-1α* and *Pgc-1β* mRNAs in isolated oocytes or whole ovarian samples were assessed by RT-PCR using *β-actin* mRNA as an internal loading control for standardization. Total RNA from five MII oocytes or a single ovary was collected using the RNeasy Plus Micro kit (Qiagen) or Tri-Reagent (Sigma-Aldrich), respectively, and reverse transcribed (Superscript II; Life Technologies) using random primers (Promega). The cDNA was amplified through 40 cycles of PCR using Buffer D (Epicentre Biotechnologies) and Taq polymerase (Life Technologies) with gene-specific primers (Table S1).

Protein Analysis. Expression of PGC-1 protein in oocytes was assessed in paraformaldehyde-fixed, paraffin-embedded ovarian tissue sections after antigen retrieval using a 1:200 dilution of rabbit anti–PGC-1 antibody (Calbiochem), essentially as described (11). Ovarian protein lysates were prepared using a dounce homogenizer in Nonidet P-40 lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA (pH 8.0)] containing phenylmethylsulfonylfluoride and a protease inhibitor mixture (Sigma-Aldrich). Homogenates were centrifuged at 13,000 × *g* for 10 min at 4 °C. Supernatants were collected and protein concentrations determined using the DC protein assay (BioRad). Protein samples (10 μg) were prepared in sample buffer, heated at 70 °C for 10 min, resolved through 4–12% Bis-Tris gels (Invitrogen), and transferred to PVDF membranes (Millipore). After blocking (5% nonfat dry milk in PBS, 0.05% Tween-20), the blots were probed with antibodies against PGC-1 (Calbiochem) followed by actin (Neomarkers) as a loading control. The blots were then washed and incubated with appropriate HRP-conjugated secondary antibodies. Detection was performed with the Amersham ECL Plus kit (GE Healthcare).

Data Assembly, Presentation, and Analysis. All experiments were independently replicated at least three times using different mice for each replicate (see legends for Figs. 1–5 and Figs. S1–S7 for additional details). Where possible, assignment of mice to each treatment group was made randomly. In some cases, each experimental replicate represents analysis of oocytes, ovaries, or vaginal smears collected from a single mouse of a given treatment group and/or genotype. In other cases, each experimental replicate represents oocytes pooled together from more than one mouse of a given treatment group and/or genotype. In either case, the approach was repeated at least two more times on different days using different mice each time (n = 3 or more mice per experimental endpoint). Samples (oocytes and ovaries) derived from a single mouse of a given treatment group and/or genotype were never split into two or more pools and then used as different replicates of a given experiment. Quantitative data from the independent experimental replicates were combined and are presented as the mean ± SEM. Statistical comparisons between mean values were performed using ANOVA followed by Student's t test. P values <0.05 were considered significant. Representative images of ovarian histology and outcomes from the chromosomal, immunodetection, mitochondrial, and gene expression analyses are provided for qualitative assessment.

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Fig. S1. CR has no effect on preimplantation embryonic development following IVF. (*A* and *B*) Percentage of cumulus cell-denuded MII oocytes (*A*) or cumulus-enclosed oocytes (*B*) collected from 3M AL-fed, 12M AL-fed, and 12M CR–AL-fed female mice that developed to two-cell stage embryos (2CE) following in vitro fertilization, and the percentage of 2CE or total inseminated oocytes (TIO) that developed to blastocyst stage (B) embryos [B(2CE) and B(TIO), respectively]. Data are the mean ± SEM of the following: (*A*) n = 55–140 denuded MII oocytes from three independent experiments using a total of 6–9 mice per group; (*B*) n = 38–144 cumulus–oocyte complexes from three independent experiments using a total of 5–7 mice per group.

Fig. S2. Relationship of oocyte yield to body weight. (*A* and *B*) Assessment of body weight versus superovulated oocyte yield in 3M AL-fed (*A*), 12M AL-fed (*B*), and 12M CR–AL-fed (*C*) females on a mouse-by-mouse basis.

Fig. S3. PGC-1 is expressed in oocytes. Immunohistochemical detection of PGC-1 (brown reaction product against blue hematoxylin counterstain) in young adult mouse ovaries. *Insets* show magnified images of typical positive oocytes.

Fig. S4. Mice lacking PGC-1α exhibit a diminished ovarian reserve with age. Number of nonatretic quiescent (primordial) and early growing (primary, preantral) immature follicles per ovary in 3M AL-fed, 12M AL-fed, or 12M AL–CR-fed wild-type (WT) or PGC-1α–deficient (null) female mice. Data are the mean ± SEM (n = 4–12 mice per group; *P < 0.05 versus 3M AL-fed females of either genotype).

Fig. S5. PGC-1 levels are comparable in ovaries of young and aged female mice. (*A*) Western blot analysis of endogenous PGC-1 protein levels in ovaries of young (3M) AL-fed, aged (12M) AL-fed, and aged (12M) CR–AL-fed females (samples prepared from three different mice are shown for each group). Pan-actin (ACTIN) was used as a loading control. (*B*) Examples of immunohistochemical detection of PGC-1 (brown reaction product against blue hematoxylin counterstain) in ovaries of the same females that were used to obtain samples for PGC-1 Western blotting (*A*).

Fig. S6. Effect of dietary manipulation on body weight. Body weight of female mice just before initiation of the CR diet (3M), upon completion of the CR regimen (11M), and 1 mo following the resumption of AL feeding (12M). Data shown are the mean ± SEM from analysis of 5–23 mice per group (*P < 0.05 versus 3M AL-fed females in each respective group). JAX, C57BL/6 mice from The Jackson Laboratory; NIA, C57BL/6 mice from the National Institute on Aging; *Pgc-1α*, mutant mouse line obtained from B. M. Spiegelman (Harvard Medical School, Boston, MA) (1).

Fig. S7. Aging-related disruption of the female reproductive cycle is prevented by CR. Proportion of aged (12M) AL-fed, and CR–AL-fed females that exhibited a typical 4- to 5-d estrous cycle or atypical estrous cycles lasting longer than 5 d. Data are from analysis of 10–15 mice per group analyzed in parallel by daily vaginal smears over a 30-d period.

Table S1. Sequence information for primers used to detect *Pgc-1α*, *Pgc-1β*, and *β-actin* mRNA in oocytes and ovaries

NM_008904	*Pgc-1α* forward	5′ TCCTCTGACCCCAGACTCAC 3′
	Pgc-1α reverse	5′ TAGAGTCTTGGAGCTCCT 3′
NM_133249	*Pgc-1β* forward	5′ AACCCAACCAGTCTCACAGG 3′
	Pgc-1β reverse	5′ ATGCTGTCCTTGTGGGTAGG 3′
NM_007393	*β-Actin* forward	5′ GATGACGATATCGCTGCGCTG 3′
	β-Actin reverse	5′ GTACGACCAGAGGCATACAGG 3′