

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. All mice were kept in the University of California, San Francisco (UCSF) animal facility with controlled temperature conditions of 21–23°C, a 12 L:12 D cycle, and ad libitum access to water and food.

Quantitation of ATP

Individual blastocyst ($n = 63$ in FB group, $n = 63$ in 5% IVF and $n = 35$ in 20% IVF) were rapidly frozen to -80°C in about $2\ \mu\text{l}$ KSOM. Solutions were prepared, and the protocols carried out according to the instructions of the adenosine 5'-triphosphate (ATP) bioluminescent somatic cell assay kit (FLASC, Sigma Chemical Co., St Louis, MO, USA). A standard curve containing 10 ATP concentrations from 0.2 fmol to 100 fmol was generated for each series of analyses. Single embryo samples were prepared by adding $50\ \mu\text{l}$ 1x Somatic Cell ATP Releasing Reagent. A volume of $100\ \mu\text{l}$ of ATP Assay Mix Working Solution was added into a reaction vial in an opaque 96-well plate and kept at room temperature for 3–5 min to allow endogenous ATP hydrolysis. Then $50\ \mu\text{l}$ ATP standard solutions for every concentration and $50\ \mu\text{l}$ sample prepared were added to the reaction vial and immediately measure the amount of light emitted with a luminometer luminescence (SpectraMax L Microplate Reader). As negative control vial with ultrapure water and no samples were used.

Mitochondrial membrane potential

Mitochondrial membrane potential was measured in living blastocysts ($n = 45$ in FB group, $n = 61$ in 5% IVF and $n = 32$ in 20% IVF) using the JC-1 assay kit (Abcam, Cambridge MA) according to the manufacturer's instructions. Lyophilized JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetramethylbenzimidazol carbocyanine iodide) was reconstituted in DMSO (1 mM), aliquoted and stored at -20°C .

Embryos were incubated in KSOM containing $1.5\ \mu\text{M}$ JC-1 for 25 min. Then the embryos were washed three times with KSOM and transferred to KSOM with 6 mg/ml HEPES under light mineral oil for fluorescence photography. As negative controls, mouse oocytes were incubated with JC-1 with or without $40\ \mu\text{M}$ of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP Abcam) for 10 min.

Samples were imaged with a confocal microscope (Leica SP5, Germany), using the $\times 20$ objective with excitation/emission wavelengths: 540/570 nm and 590/610 nm. Optically sectioned images of the samples were obtained; each section had a thickness of $7\ \mu\text{m}$, with 10 total sections (5 on either side of the embryo's equator) visualized for each embryo. Acquired images were analyzed using Image J software (NIH, MD, USA). The ratio of RITC (J-aggregate, red) to FITC (J-

monomer, green) staining was determined for the entire embryo compressed from all sections.

ROS levels

Embryo ROS was assessed with CellRox green reagent (Thermo Fischer Scientific, Waltham MA). The protocol was performed according to manufacturer's instructions. In brief, blastocysts ($n = 28$ in FB group, $n = 32$ in 5% IVF and $n = 27$ in 20% IVF) were incubated with $5\ \mu\text{M}$ CellRox green in KSOM under mineral oil for 30 min at 37°C . After incubation, embryos were washed three times with KSOM and fixed in 4% paraformaldehyde for 15 min. Then blastocysts were stained with $10\ \mu\text{g/ml}$ Hoechst 33258 for 5 min at room temperature. And then washed three times with PBS with PVP, blastocysts were mounted with Antifade Mounting Medium (Boster, Pleasanton, CA).

For a positive control, morulae were treated with $5\ \mu\text{M}$ menadione (which generates ROS) for an hour and then washed three times in KSOM and continued to be cultured for 20 h for further evaluations of ROS, as described previously (de Assis *et al.*, 2015).

Images were captured with a confocal microscope (Leica SP5, Germany), using the $\times 65$ oil objective with excitation/emission wavelengths: 485/520 nm. Five planes were obtained for every blastocyst. Quantification of average pixel intensity was performed using Image J software.

Light microscopy (LM) and transmission electron microscopy (TEM)

The following parameters were evaluated by LM and TEM and taken into consideration for qualitative assessment of the ultrastructural preservation of mitochondria: general features (shape and dimensions); integrity of the cell membrane; type and quality of the organelles; characteristics of the nucleus; presence and extent of cytoplasmic vacuolization; mitochondria number; hooded mitochondria number; ratio hooded mitochondria/normal mitochondria (Motta, *et al.* 1995, Motta, *et al.* 2000, Nottola, *et al.* 2016, Nottola, *et al.* 2006).

Assessment of mitochondria numerical density

Mitochondrial numerical density was evaluated as previously reported (Leoni *et al.*, 2015). Briefly, 3–5 embryos per group were imaged using TEM on at least five equatorial sections (distance between the sections: 3–4 μm). Values are expressed as mitochondrial numerical density per $3800\ \mu\text{m}^2$ of the embryo area. Normal and hooded mitochondrial counting was performed through collection of low-magnification TEM microphotographs of different embryos. Digital images were further enlarged, in order to easily recognize and count

mitochondria. The total number of mitochondria was then calculated (normal + hooded mitochondria) (Leoni *et al.*, 2015).

MtDNA copy number

MtDNA copy number was assessed in single blastocyst as described (Gonzalez-Hunt *et al.*, 2016). Briefly, single blastocysts were washed with KAA medium, loaded individually into 0.2 ml siliconized tubes with less than 2 μ l KAA medium and stored at -80°C until use. Genomic DNA was isolated from each sample using the ArcturusTM PicoPureTM DNA Extraction Kit (applied Biosystems), according to the manufacturer protocol: 10 μ l of extraction buffer was added to the tube, vortexed gently and incubated at 65°C for 3 hours and at 95°C for 10 min (to inactive Proteinase).

To prepare the quantification standards, a long 1186-bp fragment in the 12SrRNA region of mtDNA was amplified from mouse liver by PCR using the primer pair 5'-ACA CCT TGC CTA GCC A-3' and 5'-TTT GCC ACA TAG ACG AGT T-3'. The PCR product was extracted from the DNA Clean & Concentration-5 Kit (ZYMO RESEARCH) and subcloned into pCRTM2.1-TOPO, the plasmid DNA was purified by using QIAprep Spin Miniprep Kit (Qiagen). It was then quantified by spectrophotometry and diluted at 1×10^7 copies/ μ l. To simulate single blastocyst samples, 10 μ l of standard stock was extracted and serially diluted for use in the standard curve. Real-Time PCR, using iQSybr Green on the BioRad ICycler and the primer pair 5'- GCAATGAAGTACGCACACAC-3' and 5'- ATCTCCTCT CATAACGGATGTC-3', was performed on a iQTM SYBY Green

Supermix. The PCR program employed an initial step of 95°C for 5 min followed by 45 cycles of 15 s at 95°C for denaturation, 30 s at 60°C for annealing, and 30 s at 72°C for elongation. Melting $55-95^{\circ}\text{C}$. All samples and standards were measured in triplicate.

RT qPCR of selected genes

Blastocysts developed *in vivo* or *in vitro* (KAA with 5% oxygen or KAA with 20% oxygen) were collected as described above for detection of mtDNA, and quantitative real-time PCR was conducted on four independent biological replicates containing ten pooled blastocysts. Total RNA was extracted using a PicoPure RNA isolation kit (Arcturus, Sunnyvale, CA, USA) with DNase digestion (RNase-free DNase Set, Qiagen) to remove residual DNA. cDNA was synthesized (iScript cDNA synthesis kit, Bio-Rad) from RNA pools corresponding to ten embryo equivalents, and quantification of all gene transcripts was performed with SyBr green PCR supermix (Bio-Rad) using 0.2 embryo equivalents of cDNA from each treatment group per reaction, and was normalized according to the expression the histone variant H2A. All RT-PCR reactions were conducted in duplicates. Data were analyzed within the log linear phase of the amplification curve obtained for each primer using the comparative threshold cycle method (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycle conditions used are as follows: 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec using 0.20 embryo equivalent of cDNA in a final reaction volume of 25 μ l.

Primer sequence is provided in Supplementary Table S1.