Advanced maternal age perturbs mouse embryo development and alters the phenotype of derived embryonic stem cells

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Short title: Advanced maternal age alters stem cells

Abstract

Advanced maternal age (AMA) is known to reduce fertility, increase aneuploidy in oocytes and early embryos and lead to adverse developmental consequences which may associate with offspring lifetime health risks. However, investigating underlying effects of AMA on embryo developmental potential are confounded by the inherent senescence present in maternal body systems further affecting reproductive success. Here, we describe a new model for analysis of early developmental mechanisms underlying AMA by the derivation and characterization of mouse embryonic stem cell (mESC-like) lines from naturally-conceived embryos. Young (7-8 weeks) and Old (7-8 months) C57BL/6 female mice were mated with young males. Preimplantation embryos from Old dams displayed developmental retardation in blastocyst morphogenesis. mESC lines established from these blastocysts using conventional techniques revealed differences in genetic, cellular and molecular criteria conserved over several passages in standardised medium. mESCs from embryos from AMA dams displayed increased incidence of aneuploidy following Giemsa karyotyping compared with those from Young dams. Moreover, AMA caused an altered pattern of expression of pluripotency markers (*Sox2*, OCT4) in mESCs. AMA further diminished mESC survival and proliferation and reduced the expression of cell proliferation marker, Ki-67. These changes coincided with altered expression of the epigenetic marker, *Dnmt3a* and other developmental regulators in a sex-dependent manner. Collectively, our data demonstrate the feasibility to utilise mESCs to reveal developmental mechanisms underlying AMA in the absence of maternal senescence and with reduced animal use.

Key words: Advanced maternal age; mouse embryonic stem cells; blastocyst

Introduction

Whilst there is a global trend for women to delay bearing children, several studies indicate that advanced maternal age (AMA) leads to reduced ovarian reserve and infertility [1] and increased risk of pregnancy complications including gestational diabetes [2], preterm birth, hypertension and pre-eclampsia [3-5]. Moreover, AMA has been shown to adversely affect human offspring cardiometabolic and mental health [6,7].

The loss of ovarian reserve with AMA is common in mammals and may coincide with increased aneuploidy due to a range of segregation errors during meiosis affecting spindle dynamics [8-10]. However, maternal senescence also affects physiological condition across cell types and organs; for example, uterine decidualisation is diminished by AMA in human and mouse, affecting placental activity and increasing pregnancy loss [11,12]. Moreover, senescence from AMA may promote a chronic maternal immune response affecting diverse fetal organ function and health [13].

Given the confounding influence of maternal systemic senescence on AMA pregnancy success, models are required to understand the developmental potential of AMA oocytes and embryos and whether influenced or not by the extent and character of meiotic aneuploidy. Human and mouse embryos show retarded development in AMA [14,15]. Moreover, where AMA blastocysts are transferred to young dams, offspring growth and cardiometabolic health are adversely affected [15], indicating inherent compromised potential independent of maternal senescence and thereby demonstrating AMA contributes to the concept of the Developmental

Origins of Health and Disease (DOHaD) concept [16]. This is supported by an altered transcriptome and signalling network expression detected in AMA human blastocysts [17].

In the current study, we assess the effect of AMA on mouse blastocyst development and establish a mouse embryonic stem cell (mESC) model for investigating AMA effects on incidence of aneuploidy and developmental potential. We believe this to be the first characterisation of mESCs directly from unstimulated naturally-conceived aged embryos although pluripotent stem cells have been generated from aged mouse artificially-activated oocytes forming parthenogenetic embryos (pES cells) [18]. Also, mESCs have been generated from an assisted reproductive treatment (ART) model using mothers at different ages subjected to a combination of ART processes (maternal superovulation, *in vitro* fertilisation [IVF], extended culture to blastocyst) [19]. We find AMA mESCs display a range of aneuploidic states and accompanying perturbation in cellular and expression characteristics that permit their exploitation for underlying mechanisms influencing development within the DOHaD context. To support these primary outcomes from ageing using male mESCs, we also include separate analysis of a secondary question, to assess differences in male versus female lines following ageing.

Methods

Animal procedures

Animal procedures were conducted under UK Home Office project license in accordance with Animal (Scientific Procedures) Act 1986 and local ethics committee at the University of

Southampton (UoS). We used Young (7-8 weeks) and Old (7-8 months) C57BL/6 females (UoS Biomedical Research Facility) to investigate AMA and these were naturally mated with sexually mature CBA males (10-15 weeks, Charles River Lab, UK). This protocol permitted consistency with our previous mouse AMA study showing AMA induced postnatal health changes [15] and the cross combined the efficiency of C57Bl/6 inbred strain for derivation of ESCs with increased hybrid vigour to generate genetically identical embryos with improved stability, as shown previously [20]. Animals were housed on a controlled 07:00-19:00 h light cycle at 21°C and fed standard laboratory chow (Special Diet Services, Essex, UK) and water *ad libitum*.

Embryo collection and developmental staging

The morning after mating, dams were checked for a copulation plug to confirm successful mating. Midday on day after mating was classified as E0.5. At E3.5, C57BL/6 females were culled by cervical dislocation; dissected uterine horns were immediately placed in pre-warmed saline solution (BR0053G, Oxoid, UK) and the embryos flushed out in warm H6 medium supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma, A3311) [21]. Collected embryos were transferred to fresh dishes containing 30 µl embryo culture medium, KSOM (Potassium supplemented simplex optimized medium) with amino acids [22], and washed three times in these drops covered with a layer of mineral oil (Sigma Aldrich).

To evaluate effect of AMA on *in vivo* development, embryos derived from ten Young and Old dams were staged for development up to blastocyst and extent of blastocyst cavity

expansion (early, mid or late blastocysts). Mid-expanded blastocysts were washed in H6-BSA and used for embryonic stem cell (mESC) derivation.

Derivation and culture of mESC clones from E3.5 blastocysts

Mid-expanded blastocysts were transferred to freshly-prepared mouse embryonic fibroblasts (MEFs), mitotically inactivated with 10 µg/ ml mitomycin-C (MMC, Fisher Scientific, 10182953) in mESC medium [knockout-DMEM supplemented with 15% knockout serum replacement (KO/SR) (Gibco, 10828), non-essential amino acids (Gibco, 11140), 1 mM sodium pyruvate (Gibco, 11360), 100 µM 2-mercaptoethanol (Sigma, M7522), 2 mM glutamine, penicillin (50 U/ml)/ streptomycin (50 µg/ml) (Gibco, 10378) and 1000 U/ml of leukemia inhibitory factor (LIF)], and incubated undisturbed for 48 h under standard humidified conditions of 37°C in 5% CO_2 in air.

Blastocysts from different dams were plated onto different dishes where each blastocyst gave rise to an individual stem cell line. A total of 90 mid-stage blastocysts were utilized to derive 38 mESC lines. By the fourth day post plating of blastocysts from same dam on a single plate, inner cell mass (ICM) outgrowths could be observed to be hatched from the zona pellucida and attached on the MEFs. ICM clumps were enzymatically isolated using trypsin: ethylenediaminetetraacetic acid (EDTA) (0.05%; Gibco, 25300). Each isolated ICM outgrowth was collected in a different well of a 96-well U-shaped plate containing 50 µl/ well pre-warmed trypsin-EDTA, followed by careful pipetting up and down to achieve a single cell suspension. Trypsinized outgrowths were transferred to fresh flat-bottom 96-well MMC-MEF

coated plates and mESC medium was changed daily. Round, tight clusters of cells marked the first appearance of mESC clones, typically 4-5 days after plating the cells. Mouse ES colonies were cultured in mESC medium, passaged every 3-4 days by enzymatic dissociation and transferred to dishes with greater surface area when reaching 70% confluency. Once mESCs were expanded for 8-9 passages (P8-9; over 25-30 days), cells were either used directly or frozen at 1 x 10^6 cells/ cryovial in mESC medium with 20% KO/SR and 10% dimethyl sulfoxide, DMSO (Sigma, D5879). mESCs and MEFs were always counted and seeded in fixed proportions for each cell line and the cell numbers were kept consistent for individual experiments.

Sex analysis of derived mESC lines

MEFs were eliminated from the mESC co-culture by pre-plating on gelatin-coated dishes where they attached. After culturing the ES cells MEF-free, confluent mESCs were pelleted and treated with lysis buffer (10 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulphate (SDS) and 1 mg/ml proteinase K) and processed with NaCl followed by isopropanol [23]. Male and female mouse tail samples were used as controls. Genomic DNA concentration and quality was quantified using the Nanodrop ND-100 spectrophotometer. For sex analysis, genes *Sry* and *Zfy*, and *DxNds3 (*or *Nds3)*, located in the sex-determining regions of the Y- and X-chromosome respectively, were amplified by multiplex PCR. Amplified PCR products were run on agarose gels and bands were observed at 617 bp for *Zfy*, 404 bp for *Sry* and 244 bp for *DXNds3*. Within the Young group, 11 dams produced a total of 28 embryos that gave rise to 10 mESC lines, of which only 1 was female. Whereas, within the Old group, 13 dams produced a

total of 62 embryos resulting in 28 mESC lines with 4 being female. The male bias in sex ratio is in line with other mESC derivation studies because DNA methylation is globally reduced in XX mouse ESC lines and consequently may provide the basis for X-chromosome instability [24]. Given that the Young group generated only one female mESC line, effects of maternal age were evaluated only between male mESC lines. However, as DOHaD animal studies often display sexually dimorphic data [15], sex comparisons were also made within the Old group.

Chromosome counting (karyotyping) of mESC lines

70% confluent mESCs (P9-11) were harvested and treated with colcemid (0.1 µg/ml; Gibco, 15212-012) and incubated for 3 h at 37°C and 5% CO_2 [25]. Cells were washed, trypsinized, treated with KCl (75 mM, hypotonic solution) and fixed in the fixative solution (methanol: glacial acetic acid 3:1) before producing chromosome spreads. Slides were incubated with Giemsa stain (1:25 dilution; Sigma, G500) and analysed under a phase contrast microscope at x 100 magnification. Using Image J software, about 70 chromosome spreads were counted from each mESC line. Spreads were categorised as euploid ($2x = 40$), aneuploid ($2x \neq 40$), or polyploid ($2x \geq 80$). However, in accordance with the guidelines of American Type Cell Culture (ATCC), only karyotypically normal (>50% euploidy) mESC lines were used for further analysis. For chromosome analysis, six cell lines were screened each for Young and Old Male mESCs, as well as the Old Female mESC group.

mESC proliferation and viability assay

Established mESC lines (30,000 cells/ well; P9-11) were cultivated, harvested and seeded onto freshly prepared MMC-MEFs (27,000 cells/well) on 24-well plates in triplicates and cell counts performed at 24 h intervals up to 96 h. For counting unattached or dead cells, spent media were collected and centrifuged. Cells were counted using a Neubauer haemocytometer with viable cells detected by trypan blue (Sigma, T8154) dye exclusion assay. The mean number of cells per well was calculated at each assay time point.

Quantitative PCR (qPCR) analysis

mESCs (400,000 cells/well) were seeded on MMC-MEFs (135,000 cells/well) coated 6-well plates, cultured for 3-4 days, trypsinised and pre-plated to eliminate MEFs. RNA quality was preserved by gently mixing the derived pellet with RNA Later (Sigma, R0901). Total RNA was isolated using the RNeasy mini kit (Qiagen, UK) and quantified using the Nanodrop ND-1000 spectrophotometer. RNA integrity was analysed by the presence of clear 28S and 18S rRNA bands (in 2:1 ratio) by gel electrophoresis. First-strand cDNA was synthesized with 400 ng RNA using GoScript™ Reverse Transcription System (Promega, UK) in the DNA Engine® Peltier Thermal Cycler (BioRad, UK). The primer-templates were annealed at 25°C for 5 min, extended at 42°C for 1 h and reverse transcriptase was inactivated at 70°C for 15 min.

Primers used for specific gene targets were from Primerdesign, UK (Supplementary Table S1) and were detected using the SYBR-green 2x PrecisionPlus™ Mastermix (Primerdesign, UK) protocol. Product was amplified using Chromo4 Real-time Detector

(BioRad, UK) and analysed with Opticon Monitor v3.1 software. The amplification program included enzyme activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing (and data collection) at 60°C for 1 min, and a final extension step at 72°C for 10 min. Melting curves were generated to confirm target-specific amplification by fluorescence detection between 60°C and 95°C at 1°C steps, holding for 15 s before the program was terminated. Target genes were normalised to stable house-keeping genes [26] using Primerdesign geNorm kit [27]. Gene stability was determined using qbase+ software and genes *Ywhaz* and *Rpl13a* were identified as the most stable reference genes. Negative RT and water controls did not show amplification in the quantitation graphs. PCR products and primers were qualitatively analysed by gel electrophoresis and showed single bands at correct amplicon size.

Flow cytometry

Data acquisition was performed on FACSCalibur flow cytometer (BD Biosciences) and analyzed by FlowJo v.10 software. Fluorescence-activated cell sorting (FACS) was used to analyze the protein expression of (i) pluripotency (OCT4, NANOG and SOX2), (ii) proliferation (Ki-67), and (iii) apoptosis (Annexin V) and cell death (propidium iodide, PI) markers (antibodies used are listed in Supplementary Table S2) in undifferentiated mESCs (P9-P10) seeded on MMC-MEFs (135,000 cells/ well of a 6-well dish). In addition to using mESC-specific markers, MEF cells were further eliminated by adjusting the forward scatter (FSC, indicating cell size) and side scatter (SSC, indicating cell granularity) plots. While pluripotency analysis was conducted after culturing mESCs (200,000 cells/well) for three days; samples for cell proliferation (mESCs seeded at 150,000 cells/well) were collected every 24 h for 96 h. Cells

were stained for intracellular (nuclear) markers using Foxp3 Fixation/ Permeabilization working solution (Thermo Fisher Scientific, UK). For analysis, samples were re-suspended in flow cytometry staining buffer (PBS with 3% fetal bovine serum, FBS). Samples for cell apoptosis were prepared following eBiosciences AnnexinV-FITC Apoptosis kit protocol (BMS500FI) and mESCs (100,000 cells/well) were harvested every 24 h for 96 h. Anti-tumour agent etoposide (20 µg/ml, incubated for 4 h) induced apoptosis in Annexin V+ control samples, while floating cells were used as positive control for dead cells (PI+). The percentage of apoptotic cells was determined by generating a dual-colour dot plot (Annexin V-FITC (FL-1) vs. PI (FL-2)) and then setting a quadrant marker based on unstained and single-labelled control samples. The percentage of cells in each population and gate of interest were quantified for 20,000 cell events.

Immunocytochemistry

mESCs (30,000 cells/ well) were cultured on MMC-MEFs (27,000 cells/ well) (on sterile coverslips) in 24-well plates. At 70% confluency (3 days in culture), cells were fixed with 4% paraformaldehyde (PFA) and permeabilized in 0.2% Triton X-100 in PBS at room temperature. Non-specific antibody binding was blocked with 3% BSA and cells were stained with primary antibody at 4°C overnight. Cells were incubated with appropriate Alexa-Fluor conjugated secondary antibody in the blocking solution, for 1 h at room temperature (Supplementary Table S2). Cell nuclei were counterstained with DAPI pre-diluted in Mowiol mounting media. Images were captured using a Leica DM5000 B fluorescence microscope coupled to a Leica DFC300FX camera.

Statistics

For experimental analyses, four mESC lines derived from different blastocysts were used for AMA and control treatments. All lines were derived from separate mothers except two lines from Young male and Old female treatments which were from the same dam. Statistical analysis was performed using Statistical Package for the Social Sciences (i.e. IBM SPSS Statistics 24) with mother as a random variable for relevant sections comprising pluripotency expression, cell proliferation assays, cell death and apoptosis assays and qPCR assays. SPSS analysis showed dam origin had no significant effect. An independent Student's t-test was also used for embryo development, mESC derivation efficiency, sex differences and karyotype aberrations. Pairwise comparisons were made between Young male v Old male for effect of AMA on male embryo derived mESCs, and between Old male v Old female for effect of sexual dimorphism within the Old group with outcomes from both separate comparisons integrated into the same graphs for conciseness. Data were tested for assumptions of normality using the Shapiro-Wilk normality test (GraphPad Prism v.8 software) and considered normal where values were over significance cut off ($P > 0.05$). Variance homogeneity was analysed using the F-test. All data are expressed as means ± Standard Deviation (SD). $P<0.05$ was considered statistically significant and $P<0.1$ was considered as a non-significant trend towards significance.

Results

Advanced maternal age (AMA) delays embryo development at E3.5

At E3.5, embryos were collected from Young (7-8 weeks) and Old (7-8 months) C57/BL6 mothers and developmental stage assessed (Table 1). Whilst no difference was observed between Young and Old mice in dams becoming pregnant, embryo development was delayed in Old mothers with fewer blastocysts formed ($P<0.05$), more present at an earlier stage of expansion ($P<0.05$), and at non-significant trend for more morulae and arrested/degenerate embryos ($P<0.1$) compared with Young mothers (Table 1) (Figure 1).

AMA did not affect derivation efficiency of mESC lines

Mouse ESC lines were derived from E3.5 blastocysts of Young and Old dams and cultured on MMC-MEFs in mESC medium. ICM outgrowths were cultured individually and each mESC line was generated from an individual blastocyst. No differences were found in the derivation efficiency of mESC lines isolated from individual Old dams (45%) compared to Young dams (36%). The number of male mESC lines (86 % in Old; 90% in Young) isolated was higher than female lines for both groups, hence effects of maternal age were evaluated only between male mESC lines. However, sex comparisons were made within the Old group.

mESC lines from Old mothers show chromosomal abnormalities

For karyotype analysis, 70% confluent mESC lines (P10-11) were treated with colcemid and hypotonic solution (KCl), and slides stained with Giemsa. From each mESC line, at least 70 chromosome spreads were counted and categorised as euploid ($2x = 40$), aneuploid ($2x \neq 40$), or polyploid ($2x \geq 80$) and only those lines with more than 50% euploidy were selected for further experiments. Since mESC karyotype may vary with extended in vitro culture [28] and

lines with apparent normal karyotype (>50%) may have a specific aneuploidy with a higher number of spreads with 38, 39 or 41 chromosomes [29], aneuploid spreads were further grouped into $2x \leq 37$, $2x = 38$ and $2x = 39$ (Table 2). Old male mESCs showed reduced euploidy and consequently increased total aneuploidy ($2x \neq 40$, $P = 0.024$) than Young male lines (Table 2). Old male mESCs had increased number of spreads with less than 37 chromosomes ($2x < 37$, $P = 0.008$), with 37 ($2x = 37$, $P = 0.033$) and, at non-significant trend, with 38 ($2x = 38$, $P = 0.090$) chromosomes, compared to Young male mESC lines. No differences were found between polyploidy levels ($2x \geq 80$). Separated by sex, Old male lines showed greater number of cells with 38 chromosomes ($2x = 38$, $P = 0.03$) compared to Old female lines, although no differences were observed between euploidy (Table 2).

Effect of AMA on pluripotency and differentiation marker expression of derived mESCs

Pluripotency of stem cells is typically divided into naïve and primed states [30]. Collectively, AMA did not affect the relative mRNA expression of the naïve pluripotency markers *Oct4*, *Nanog* and *Lif* but Old male mESCs showed reduced *Sox2* expression ($P=0.049$) compared to Young male lines (Figure 2A). Flow cytometry assisted intracellular (nuclear) staining revealed no significant effect of AMA on the pluripotent protein expression (i.e. OCT4, NANOG, and SOX2) of the Old male and Young male mESC lines after 4 days culture (Figure 2B). However, OCT4 expression appears dynamically regulated over 0-96 h culture with a higher proportion of Young male cells expressing OCT4 up to 48 h ($P <0.001$) compared with Old male lines, and while both lines diminish OCT4 expression at 72 h, this is slower in Old male lines ($P <0.05$; Figure 2C). Sexually dimorphic differences were observed within the Old group, where the Old

303 Female mESC lines displayed reduced NANOG-expressing mESCs (P=0.033) compared to the
304 Old Male group (Figure 2B).
305
306 Maternal age did not influence the gene expression of mesodermal (*Brachyury*) or
307 endodermal (*Gata4*) transcripts in cell lines maintained in undifferentiated state (Figure 2A).
308 However, Old male mESC lines had, a non-significant trend for reduced transcript levels of
309 *Fgf10* (P=0.0573), important in cardiomyocyte differentiation, and *Tgf-α* (P=0.0522), important
310 in regulating cell proliferation and apoptosis (Figure 2A) but no differences in other signalling
311 and proliferation markers (*mTOR*, *Gsk3b*, *Akt1*, *Mapk1*). No sex-induced differences were
312 observed within the Old mESC group for these markers (Figure 2A).
313
314 For qualitative analysis, mESC lines were cultured at the same starting density for 4 days
315 (as used in FACS) before immunocytochemistry for OCT4, SOX2, BRACHYURY and Dapi
316 nuclear staining (Figure 2D-F). Strong expression of OCT4 and SOX2 was present in both
317 Young and Old male lines (Figure 2D, E). Old Female mESC lines however, showed relatively
318 weak staining and smaller colonies compared to Old Male lines. Old Male and Female lines
319 displayed similar staining for BRACHYURY which was not detected in Young male mESC lines
320 (Figure 2F).
321
322 *Effect of AMA on cell proliferation and viability of derived mESCs*
323 Cell lines were seeded (27,000 cells/well) on 24-well plates for 96 h and harvested every 24 h.
324 Phase contrast images indicated Young male lines (Figure 3A) to have more and larger colonies

at all time points compared to Old male mESC lines (Figure 3B), whereas Old Female mESC lines (Figure 3C) had the smallest and least number of colonies at all time points. For analysis, mESCs were separated into unattached (dead) and adhered cells at each time point; adherent cells were trypsinized and stained with viability dye, trypan blue (TB) to distinguish live (TB negative) and dead (TB positive) cells. Young male mESC lines had a higher proportion ($P=0.001$) and increased number ($P<0.001$) of live adherent cells compared to Old male mESC lines at all time points (Figure 3D). Consequently, Young male lines had fewer unattached (dead) cells ($P<0.001$) compared to Old male lines at 48 h ($P= 0.0004$) and 72 h ($P= 0.015$) (Figure 3E). Relative to Old males, Old female lines had an increased proportion of live cells at 48 h ($P= 0.0493$) with a consequent decrease in unattached/dead cells (Figure 3D, E). All groups showed increased rate of cell growth ($P<0.001$) over the first 3 days of culture, before reducing by day 4, as expected. (Figure 3D).

Proliferation was further assessed over 24-96 h culture by the level of nuclear-specific biomarker of cellular growth, Ki67 [31] in viable cells using FACS (Figure 3F). In agreement with the direct proliferation analysis, Ki67 expression was increased in Young male mESC lines compared to Old male mESC lines at 24 h ($P = 0.01$) and 48 h ($P = 0.001$; Figure 3F) while sex-associated differences were not observed within the Old mESC lines.

Cell apoptosis and death analysis of derived AMA mESCs

We assessed whether the reduced proportion of live cells and proliferation in Old male mESC lines was contributed to by increased apoptosis or cell death assessed by protein expression of

Annexin V- FITC and PI using FACS. Cell lines were seeded at defined cell density and analysed every 24 h for up to 96 h. Cells were selected from combined mESCs and MEFs using the pluripotency surface marker, Stage-Specific Embryonic Antigen-1 (SSEA-1) which did not differ between Young and Old male lines (data not shown). SSEA-1 positive cells were further separated into Annexin V and PI negative (live cells), Annexin V positive and PI negative (early apoptotic cells), Annexin V and PI positive (late apoptotic or necrotic cells), and Annexin V negative and PI positive (dead cells). Data are presented as proportion positive of total cells (stained and unstained cells) (Figure 4). Young and Old male lines had similar proportions of live cells at each time point (Figure 4A) and also of early apoptotic cells (Figure 4B). However, differences between Old male and female lines were found including a non-significant trend for more early apoptotic cells in female lines at 24 h but fewer at later time points ($P < 0.1$; Figure 4B). Old male lines had a non-significant trend for increased late apoptotic or necrotic cells when compared to Young male lines at 24 and 48 h ($P < 0.1$) and these cells were increased in Old female lines compared to male lines ($P < 0.05$; Figure 4C). Old female lines were also increased in dead cells at 48 h compared to Old male cells (Annexin negative / PI positive; $P < 0.01$) but no differences were detected between Young and Old male lines (Figure 4D). Overall, Old Female lines had a non-significant trend for decreased live ($P = 0.095$) and significantly increased necrotic ($P = 0.003$) proportion of total cells compared to Old Male lines (Figure 4A, C).

We next assessed mRNA expression of cyclin dependent kinase inhibitors *p19* and *p21* and cysteine dependent proteases in apoptosis of initiator (*caspase 9*), executor (*caspase 3*) and

inflammatory (*caspase 1*) caspases (Figure 4E). Although no age- or sex-induced differences were observed, Old male mESCs showed a non-significant increase in mean transcript levels for all markers compared to Young male lines.

Effect of AMA on expression of epigenetic modifiers and glucose transportation

The effect of AMA on mRNA expression of epigenetic modifiers including DNA methyl transferases (*Dnmt1, Dnmt3L, Dnmt3a, Dnmt3b*) and histone deacetylases (*Hdac-1, Hdac-3*) was assessed. Old male lines showed reduced *Dnmt3a* expression ($P = 0.049$) compared to Young male lines (Figure 5A). Old male lines also had a non-significant trend for reduced expression of *Dnmt3b* compared to Old female lines ($P < 0.1$; Figure 5A). No age or sex related differences were observed for *Hdac1* or *Hdac3*.

We further analysed expression of metabolic regulators across cell lines including mRNA expression of glucose and insulin metabolizers *Gapdh*, *InsR*, *Igf1* and *Igf1R*, and glucose transporter isoforms *Glut1*, *Glut3*, *Glut4* and *Glut8* (Figure 5B). Here, Old male mESC lines had a non-significant trend for reduced glucose transporter *Glut4* levels ($P = 0.0685$; Figure 5B) while Old female lines had reduced levels of the glycolytic enzyme *Gapdh* ($P = 0.029$) and a non-significant trend for reduced glucose transporter, *Glut8* ($P = 0.055$) compared with Old male lines (Figure 5B).

Discussion

We have investigated the effects of advanced maternal age (AMA) on mouse blastocyst development and the phenotype of undifferentiated mESCs derived from the ICM. Our primary objective was to develop a model for molecular and cellular analysis of early mechanisms contributing to the poorer fertility and health outcomes known to occur, independent of maternal somatic senescence, as a consequence of AMA [1,6,7,14,15]. Our model of AMA used 7-8 month females when the decline in fertility is manifest by reduced oestrus cycles, decreased breeding performance with litter size and number of live births declining [32,33] and so is broadly comparable to ~40 year old human in terms of age-related reproductive senescence. However, whilst we recognise across different bodily systems, comparison of mouse and human senescence may be complex, with 'middle age' compared as 10-14 months versus 37-48 years, respectively[34], the current focus was on reproductive potential to ensure our model had clinical relevance.

From our previous studies on the effects of maternal undernutrition on early embryo developmental potential, we have shown that derived mESCs do indeed exhibit and retain molecular and cellular changes in phenotype after several passages that match the *in vivo* condition and facilitate understanding of early mechanisms of adverse programming of health [35,36]. This has also been shown in one study for mESCs derived from ART treatments to assess phenotypic changes[19] but in another, mESC stability did not retain ART-mediated effects which may reflect strain differences[37]. This strategy can therefore help overcome the inaccessibility and limited size of the pre- and peri-implantation embryo *in vivo* and reduce animal use in biomedical research.

Our data revealed AMA induced delay in embryo development (E3.5) but did not affect the derivation efficiency of mESC lines nor the strong bias of male over female cell lines. Female ESC lines are more prone to developing abnormal karyotypes related to reduced cellular function and proliferation [24,38]. Collectively, mESCs from male AMA blastocysts exhibited a higher incidence of aneuploidy and karyotypic abnormalities, particularly increased occurrence of < 40 chromosomes. Normal karyotype AMA lines also displayed an altered pattern of expression of pluripotency genes and evidence of increased cellular senescence with poorer proliferation and increased incidence of late apoptotic cells. Lastly, AMA mESC lines exhibited reduced expression of genes important in critical cellular processes including epigenetic, metabolic and differentiation pathways. AMA lines also exhibited differences in these criteria dependent upon sex. Thus, these new cell lines exhibit all the characteristics expected of undifferentiated mESCs, but since they have not yet been used to generate chimeras with full development of all three germ layers, in strict terms, they are mESC-like.

Whilst derivation and *in vitro* culture of mESC lines may potentially increase the risk of epigenetic abnormalities over time and increasing passage number [39,40], we consider this unlikely here since passages have been limited and culture of both Old and Young lines normalised throughout experiments. Thus, differences observed between the treatments would already be established and programmed before derivation, permitting the AMA lines to provide a model for analysis of cellular mechanisms of aging on developmental potential.

435 The reduced viability and retarded rate of blastocyst development found in the Old group
436 mirrors that shown previously [19,41]. Poorer AMA fertility can derive from a combination of
437 maternal cellular deficiencies including reduced mitochondrial efficiency and energy production,
438 increased oxidative stress and declining steroid hormone levels, all of which may contribute to
439 chromosomal and spindle errors as well as reduced early embryo viability [42-45]. Indeed,
440 microarray analysis of aged metaphase II mouse oocytes reveal significant altered expression of
441 genes involved in chromatin and spindle organisation, mitochondrial function and oxidative
442 stress [46,47] with a similar pattern evident in the human [48], and some but not all pathways altered at
443 the proteomic level [49]. Moreover, pharmacological treatments to overcome such cellular
444 deficiencies can reverse these effects of aging [50,51]. In our previous study on AMA in this strain
445 of mice, blastocysts from aged dams also showed fewer trophectoderm and total cells compared
446 with controls [15]. Collectively, these oocyte-derived perturbations continue into post-implantation
447 concepti with abnormalities in fetal and placental morphology [52].

448

449 Whilst embryos from Old mothers showed characteristic poorer quality and viability,
450 ESC derivation efficiency was similar to Young embryos. However, early passage mESCs
451 showed increased incidence of aneuploidy typical of the AMA phenotype *in vivo*. In particular,
452 Old male lines exhibited increased percentage of karyotype with 38 or less chromosomes than
453 Young male lines but no differences in incidence of polyploidy, suggesting instability to be
454 chromosome-specific, as has been shown for mESCs in general [29]. The increase in aneuploidy
455 provides authentication of the AMA phenotype known to contribute to the reduced fertility of

456 older women in ART [53]. Indeed, excess aneuploidy is also found in an ART model of mESC
457 formation and increased by maternal age [19].

458

459 Alterations in pluripotency marker expression was one notable characteristic of AMA
460 mESC lines. Whilst these lines had normal expression of the naïve pluripotency markers *Oct4*,
461 *Nanog* and *Lif*, AMA lines showed reduced *Sox2* expression and an altered profile over time of
462 OCT4 expression at the protein level, reduced in early cultures but sustained in later cultures.
463 The reduction in *Sox2* expression may reduce overall pluripotency and increase differentiation of
464 ESCs by disturbing the positive-feedback loop between core-regulatory transcription factors
465 essential in maintaining pluripotency as well as continuous ESC self-renewal [54-57]. In so doing,
466 the reduction in *Sox2* expression may contribute to the observed dynamic changes in OCT4
467 expression. Like other pluripotency factors, SOX2 not only maintains self-renewal but assists in
468 proliferation of ES cells [58], so may also contribute to the reduced proliferation and cell survival
469 found in the Old mESC lines.

470

471 Old male mESC lines showed an increased proportion of dead cells, reduced live cells,
472 reduced cell proliferation by Ki67 FACS analysis, reduced expression of the mitogen *Tgfα*, and
473 increased proportion of late apoptotic/necrotic cells by Annexin V- PI FACS assay. Although the
474 cyclin dependent kinase inhibitors (*p19* and *p21*) and apoptosis markers (*caspase 9*, *caspase 3*
475 and *caspase 1*) showed no transcriptional differences between the groups, Old male mESCs
476 showed increased mean transcript levels for all markers compared to Young male mESC lines.
477 Collectively, these data warrant further evaluation of the role of altered pluripotency expression

in this outcome as well as cell death mechanisms such as the Ras/Raf/extracellular signal-regulated kinase (*Erk*) signalling pathway [59].

The AMA male lines also showed differences in gene expression associated with other cellular processes compared to the control lines. *Dnmt3,* the key de novo DNA methylation regulator in development, is reduced in Old versus Young male lines which has implications for the epigenetic restructuring that occurs in the embryonic genome during post-implantation lineage allocation [60]. Consistent with this result, aged oocytes and embryos show reduced DNA methylation both *in vivo* and *in vitro* [61]. In stem cells, de novo DNA methyltransferases maintain self-renewal, pluripotency and, therefore, affect proliferation and differentiation capacity of these cells [62] suggesting this epigenetic deficiency may contribute more broadly to our AMA mESC phenotype. Similarly, the reduction in expression of *Fgf10* in Old male lines may affect cardiomyocyte differentiation since it plays a critical role in cardiac development in the embryo [63]. Poor cardiac function and hypertension has been shown to be symptomatic of adult offspring in response to adverse periconceptional developmental programming from diverse environments including maternal undernutrition and ART treatments [16]. Moreover, mouse maternal aging leads to increased blood pressure and reduced heart size in offspring [15].

Phenotypic differences were also observed in the AMA lines dependent upon sex. Female lines displayed reduced *Nanog* expression and showed relatively weak staining and smaller colonies compared to male lines. Female AMA lines also displayed increased incidence of apoptotic/necrotic cells than male lines and had differences in level of expression of epigenetic

and metabolic genes. Developmental programming has consistently shown differences in phenotype and outcomes between male and female offspring [16] including in response to AMA [15]. Sex-dependent differences in susceptibility to disease usually arise in utero [64]. Moreover, environmental factors are known to differentially influence cell signalling, gene expression and morphogenesis during development dependent upon sex that can be maintained into postnatal life [65].

In conclusion, we have shown that AMA leads to a retardation in preimplantation development and a complex series of phenotypic effects in derived mESC lines at chromosomal, molecular and cellular levels. These perturbations affect pluripotency, cell proliferation and apoptosis with potential to alter epigenetic and differentiative mechanisms throughout development. These novel cell lines derived from natural embryo development provide a model to investigate early mechanisms of AMA effect on developmental programming in the absence of confounding maternal senescence.

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Author contribution

PK performed experiments, analysed data, drafted and edited the manuscript.

NRS provided technical and training support, analysed data and edited the paper.

BS provided technical and training support.

MAV provided technical and training support, edited the manuscript.

JJE provided technical and training support and edited the manuscript

TPF conceived and designed the study, drafted and edited the manuscript.

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Figure legends

Figure 1. Representative images of embryos derived from (A) Young (7-8 weeks) and (B) Old (7-8 months) female mice (C57BL/6 females mated with CBA males) at E3.5. Magnification bar = 200 μm.

Figure 2. Pluripotent, differentiation and cell proliferation markers in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male, and Old Male vs Old Female (7-8 months) mESC lines. (A) Genes of interest were normalized to *Ywhaz* and *Rpl13a* within geNorm; Old male mESC lines had reduced expression (qPCR) of *Sox2* (P=0.049), and non-significant trend reduction in *Fgf 10* (P=0.0573) and *Tgf α* (P=0.0522) relative to Young male lines. **(B)** Quantitative analysis of protein expression for pluripotent markers. No differences were identified between Old Male vs Young Male lines but Old Female mESCs had reduced NANOG expression ($P<0.05$); mESCs were cultured in triplicate with n=4 mESC lines per group. **(C)** Protein expression of OCT4 over time is distinct between Old and Young Male mESCs with

reduced expression at earlier times (P<0.001) and sustained expression at later times (P<0.05) in Old Male lines. **(D-E)** Qualitative immunocytochemistry images showed similar expression of pluripotent markers OCT4 and SOX2, and colony size in Young and Old Male mESC lines. Old Female lines however, showed reduced colony size with less relative expression of the pluripotent markers compared to the Old Male group. **(G)** Immunocytochemistry showed no expression of the mesodermal marker, BRACHYURY, in Young male lines although colonies stained positive in Old Male and Old Female lines. Magnification bar = 200 µm. Data presented as means ± SDs based on n = 4 mESC lines per group examined in triplicate; **P<0.001, *P<0.05, ᐞ P<0.1 non-significant trend. Note: for **(A-C)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 3. Cell proliferation and viability of undifferentiated Young male (7-8 weeks), Old (7-8 months) male, and female mESC lines. (A-C) Representative phase-contrast images of cell proliferation of Young male, Old male and Old female mESCs. After seeding, images were captured every 24 h over a period of 96 h. Images show that Young male **(A)** mESC lines have larger colonies with increased density of cells/ colony compared to Old male **(B)** mESC lines. Old Female mESC lines **(C)** present much smaller colony size and fewer mESC clones compared to Old Male mESC lines. Arrows and parenthesis point toward the colony size differences across the groups. Magnification = 100 µm. **(D, E)** Trypan negative (live) cells are presented as proportion of adherent (live and dead) cells, and floating cells as proportion of total (live, dead

and floating) cells. **(D)** Old male mESC lines had fewer live adherent cells at all time points, and **(E)** increased proportion of floating cells at 48 h and 72 h, as compared to Young male mESC lines. **(F)** Protein expression of cell proliferation marker Ki67 over time presented as the proportion positive of total cells (stained and unstained). Ki67 expression was lower in Old vs Young mESC lines at 24 h (P=0.011) and 48 h (P=0.001). Data presented as means ± SDs based on n = 4 mESC lines per group. * P<0.01, ** P<0.001, *** P<0.0001, **** P<0.00001. Note: for **(D-F)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 4. FACS analysis for detection of early apoptosis (Annexin V) and cell death (PI) in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male, and Old male vs Old female (7-8 months) mESC lines. mESCs were cultured in duplicate. (A-D) Data is presented as proportion of total cells. Compared to the Young group, Old mESCs presented (C) increased necrosis at non-significant trend at 24 h (P=0.075) although the (B) proportion of early apoptotic cells was unaffected by age and time. Sex differences were observed within the Old mESC group where Old Female mESC lines showed (B) increased proportion of early apoptotic cells at non-significant trend (P=0.059) at 24 h, which decreased at 48 h (P=0.055) and 96 h (P=0.085) also at non-significant trend. Old Female mESC lines also exhibited increased proportion of (C) necrotic cells at 72 h (P=0.035) and 96 h (P=0.035), and (D) dead cells at 48 h (P=0.01) compared to Old male mESC lines. (E) However, RT-qPCR analysis of cell apoptotic markers

was unaffected by maternal age and sex. Data presented as means ± SDs based on n = 4 mESC lines per group, where ▵ = trending P<0.1, * = P<0.05 and ** = P<0.01. Note: for **(A-E)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 5. Relative gene expression for epigenetic modifiers and glucose transporters in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male and Old male vs Old female (7-8 months) mESC lines. Real-time PCR analysis of DNA methyltransferases *Dnmt1*, *Dnmt3L*, *Dnmt3a* and *Dnmt3b*, histone modifiers *Hdac1* and *Hdac3*; glucose transporters *Glut1*, *Glut3*, *Glut4* and *Glut8* and metabolizers *Gapdh*, *InsR*, *Igf1* and *Igf1R*. Genes of interest were normalized to *Ywhaz* and *Rpl13a* within geNorm. (A, B) Old male mESC lines showed reduced expression of *Dnmt3a* (P=0.049) and *Glut4* at non-significant trend level (P=0.0685) compared to Young male mESC lines. Old Female mESC lines showed reduced expression of *Dnmt3b* at non-significant trend (P=0.078) and increased relative expression of *Gapdh* (P=0.029) and *Glut8* (P=0.055) at non-significant trend compared to Old Male mESC lines. Data presented as means ± SDs based on n = 4 mESC lines per group. * indicates P<0.05 and ▵ indicates trend P< 0.1. Note: for **(A,B)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Table 1. Embryo development from Young and Old female mice at E3.5.

	Total number (%)	
Flushed at E3.5	**Young (7-8 weeks, n = 10)**	**Old (7-8 months, n = 10)**
Arrested/Degenerate	9 (11.0)	18 (23.7)
Morulae	4 (4.9)	13 (17.2) †
Early blastocysts	6 (7.3)	14 (18.4) *
Mid blastocysts	52 (63.4)	20 (26.3) †
Late blastocysts	11 (13.4)	11 (14.5)
Total blastocysts	69 (84.1)	45 (59.2) *

Number (%) of embryos collected; n = number of dams. * $P<0.05$; † $P<0.1$

Table 2. Effect of maternal age on karyotype of mESCs lines derived from Young and Old dams.

	Chromosome spreads (%)		
Karyotype n	**Young male** 6	**Old male** 6	**Old female** 4
Euploidy (2x = 40)	**67.4**	**41.65***	46.23
Total Aneuploidy (2x ≠ 40)	**32.6**	**58.35***	53.4
Segregation within Aneuploidy (%)			
2x < 37	**8.35**	**18.41****	14.62
2x = 37	**1.44**	**4.78***	6.08
2x = 38	**3.02**	**5.67†**	**0.12***
2x = 39	5.5	8.58	8.24
2x >40, < 80	9.24	20.35	12.13
2x = 80	3.27	1.43	0.72
2x ≥ 80	1.78	0.25	0.24

Data presented as % chromosome spreads; n = number of cell lines analysed (70 spreads from each); x = number of chromosomes; ** P<0.01; * P<0.05, † P<0.1 (Old males vs Young males; Old females vs Old males).

Supplementary Table S1. List of primer sequences used for q-PCR

Gene symbol	Forward sequence (5'- 3')	Reverse sequence (3'- 5')
Akt1	TCGTGTGGCAGGATGTGTAT	ACCTGGTGTCAGTCTCAGAGG
Brachyury	GCTTCAAGGAGCTAACTAACGAG	CCAGCAAGAAAGAGTACATGGC
Casp 1	AGGCACGGGACCTATGTGAT	AGCTGATGGAGCTGATTGAAG
Casp 8	AGCACAGAGAGAAGAATGAGCC	TTGGCGAGTCACACAGTTCC
Casp3	GACTCCACTTTCCACGCAAA	CCCACCCCCAATCATTCCT
Dnmt 1	GCTACCAGTGCACCTTTGGT	ATGATGGCCCTCCTTCGT
Dnmt 3a	ACACAGGGCCCGTTACTTCT	TCACAGTGGATGCCAAAGG
Dnmt 3b	GCCTGCAAGACTTCTTCACTACT	GGTACAACTTGGGTGGCTCA
Dnmt 3L	AACCGACGGAGCATTGAA	CCGAGTGTACACCTGGAGACT
EGF	GGGCAGGAAACAAGTTCGT	CATGCCCCACAGGATTTG
FGF-10	AACAACTCCGATTTCCACTGA	CGGGACCAAGAATGAAGACT
FGF-4	CGGAGAGAGCTCCAGAAGAC	TACCTGCTGGGCCTCAAA
Gapdh	Proprietary information (Primerdesign)	Proprietary information (Primerdesign)
Gata4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
Glut 1	ATGGATCCCAGCAGCAAG	CCAGTGTTATAGCCGAACTGC
Glut 3	TTGCCCTGAGAGTCCAAGA	ACAAGCGCTGCAGGATCT
Glut 8	CCAGCGCCACTCTAGGAC	CAGCTGATGGTTGTCACTGG
Glut-4	GTGGACTGGTTTCTGGACCG	CCTCAATCACCGAGTTCCCC
Gsk3b	GCACTCTTCAACTTTACCACTCAAG	CGAGCATGTGGAGGGATAAG
Hdac1	AATCCTGAACTGCCAAGTGC	AAGCCTGAAAAGGGGTCCTA
Hdac3	CTCTGGTGAAGGGTTTGGAA	TGTCCATGTCTCATCCCTGA
Igf-1	GAAGACGACATGATGTGTATCTTTATC	AGCAGCCTTCCAACTCAATTAT
Igf1-R	CACTTGCATGACGTCTCTCC	GAGAATTTCCTTCACAATTCCATC
InsR	TCTTTCTTCAGGAAGCTACATCTG	TGTCCAAGGCATAAAAAGAATAGTT
LIF	AGCAGCAGTAAGGGCACAAT	AAACGGCCTGCATCTAAGG
MAPK-1	CCTTCAGAGCACTCCAGAAAGT	ACAACACCAAAAAGGCATCC
mTOR	GTGGAAAAGTGCTCGGAAGA	CATGGTCTGCACAAAGTGTTG
Nanog	TGCTTACAAGGGTCTGCTACTG	GAGGCAGGTCTTCAGAGGAA
Oct4	GTTGGAGAAGGTGGAACCAA	CTCCTTCTGCAGGGCTTTC
p19	TGCTGGATTGCAGAGCAGTAA	GCATGCAGAGATTCCGAGAGA
p21	CCGAGAACGGTGGAACTTTGAC	GAACGCGCTCCCAGACGAAGTTG
Rpl13a	Proprietary information (Primerdesign)	Proprietary information (Primerdesign)
Sox2	TGGGCTCTGTGGTCAAGTC	TGATCATGTCCCGGAGGT
TGF-α	ACGTACCCAGAGTGGCAGAC	GTGCCCAGATTCCCACAC
Ywhaz	Proprietary information (Primerdesign)	Proprietary information (Primerdesign)

Supplementary Table S2. List of antibodies used.

Flow cytometry

Coupled antigen	Company	Catalog no.	Working dilution
NANOG-APC	Miltenyi Biotec	REA297	1:10
OCT3/4 Isoform A-APC	Miltenyi Biotec	REA622	1:10
SOX2-PE	Miltenyi Biotec	REA320	1:10
SSEA-1-APC	Miltenyi Biotec	REA321	1:10
Ki-67-FITC	Miltenyi Biotec	REA183	1:10
AnnexinV-FITC Kit	eBioscience	BMS500FI	Annexin-FITC 1:4 PI- 1:5

Immunocytochemistry

Antigen	Species	Company	Cat no.	Working Dilution
Nanog	Mouse	SantaCruz Biotechnology (SCBT)	293121	1:50
Oct4	Mouse	SCBT	5279	1:50
Sox2	Mouse	SCBT	365823	1:50
Gata4	Mouse	SCBT	25310	1:50
Brachyury	Mouse	SCBT	166962	1:50
Alexa 488	Goat	Abcam	A11008	1:1000
Alexa 594	Donkey	Abcam	A21203	1:1000

Figure 1

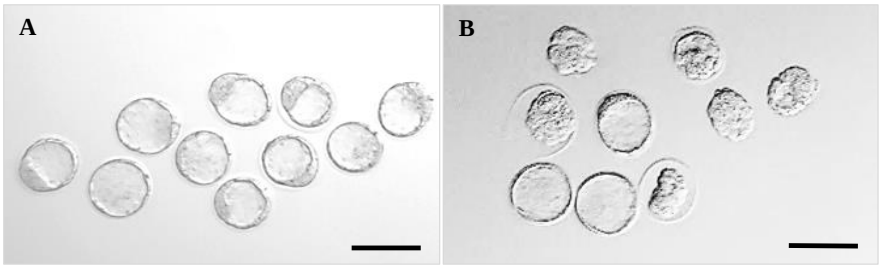

Figure 1 Representative images of embryos derived from **(A)** Young (7-8 weeks) and **(B)** Old (7-8 months) female mice (C57BL/6 females mated with CBA males) at E3.5. Magnification bar = 200 µm.

Figure 2

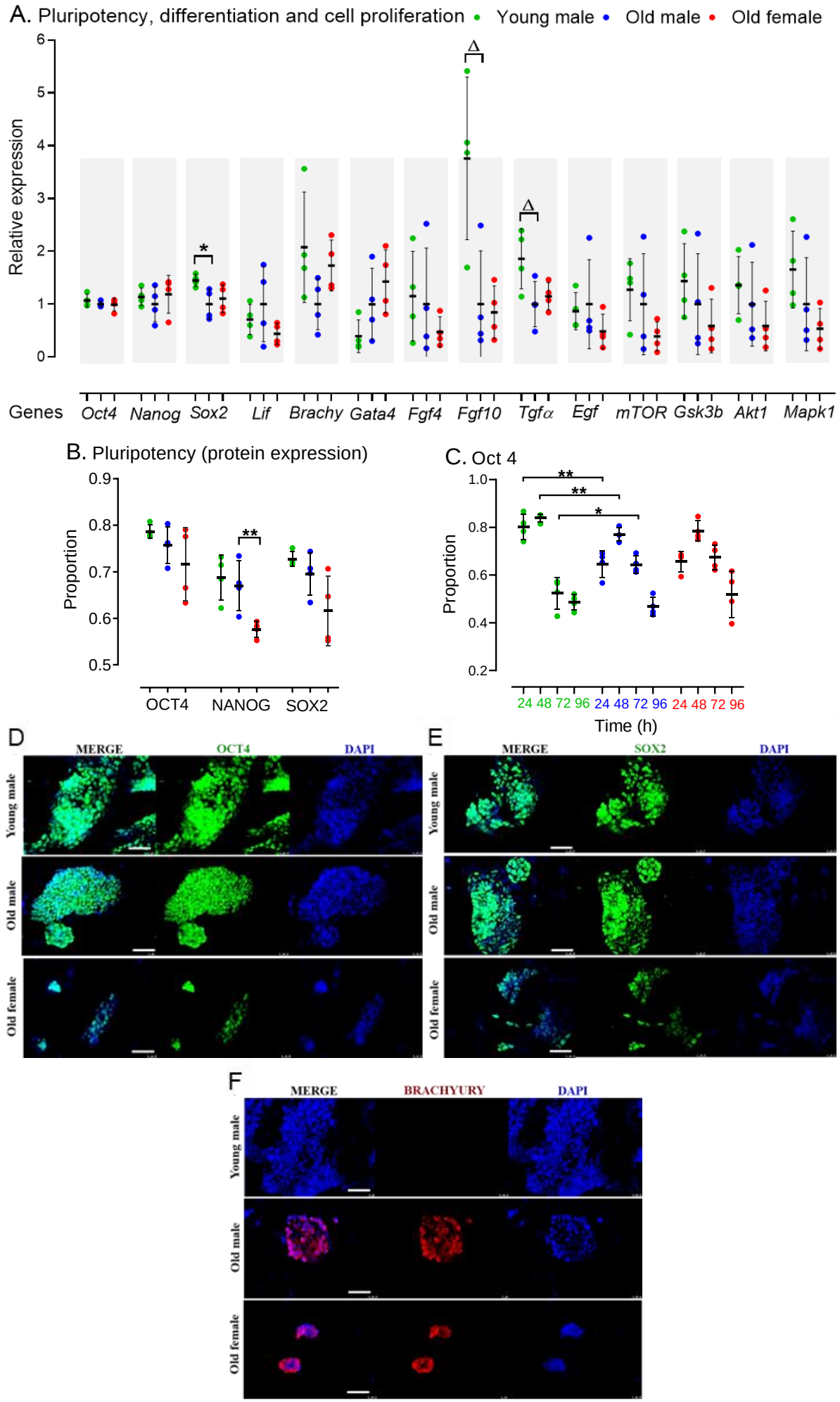
A. Pluripotency, differentiation and cell proliferation
Young male
Old male
Old female
Relative expression
Genes
Oct4
Nanog
Sox2
Lif
Brachy
Gata4
Fgf4
Fgf10
Tgfα
Egf
mTOR
Gsk3b
Akt1
Mapk1
B. Pluripotency (protein expression)
Proportion
OCT4
NANOG
SOX2
C. Oct 4
Proportion
Time (h)
D
MERGE
OCT4
DAPI
Young male
Old male
Old female
E
MERGE
SOX2
DAPI
F
MERGE
BRACHYURY
DAPI

Figure 2. Pluripotent, differentiation and cell proliferation markers in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male, and Old Male vs Old Female (7-8 months) mESC lines. **(A)** Genes of interest were normalized to *Ywhaz* and *Rpl13a* within geNorm; Old male mESC lines had reduced expression (qPCR) of *Sox2* (P=0.049), and non-significant trend reduction in *Fgf 10* (P=0.0573) and *Tgf α* (P=0.0522) relative to Young male lines. **(B)** Quantitative analysis of protein expression for pluripotent markers. No differences were identified between Old Male vs Young Male lines but Old Female mESCs had reduced NANOG expression (P<0.05); mESCs were cultured in triplicate with n=4 mESC lines per group. **(C)** Protein expression of OCT4 over time is distinct between Old and Young Male mESCs with reduced expression at earlier times (P<0.001) and sustained expression at later times (P<0.05) in Old Male lines. **(D-E)** Qualitative immunocytochemistry images showed similar expression of pluripotent markers OCT4 and SOX2, and colony size in Young and Old Male mESC lines. Old Female lines however, showed reduced colony size with less relative expression of the pluripotent markers compared to the Old Male group. **(G)** Immunocytochemistry showed no expression of the mesodermal marker, BRACHYURY, in Young male lines although colonies stained positive in Old Male and Old Female lines. Magnification bar = 200 μm. Data presented as means ± SDs based on n = 4 mESC lines per group examined in triplicate; **P<0.001, *P<0.05, ▵ P<0.1 non-significant trend. Note: for **(A-C)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 3

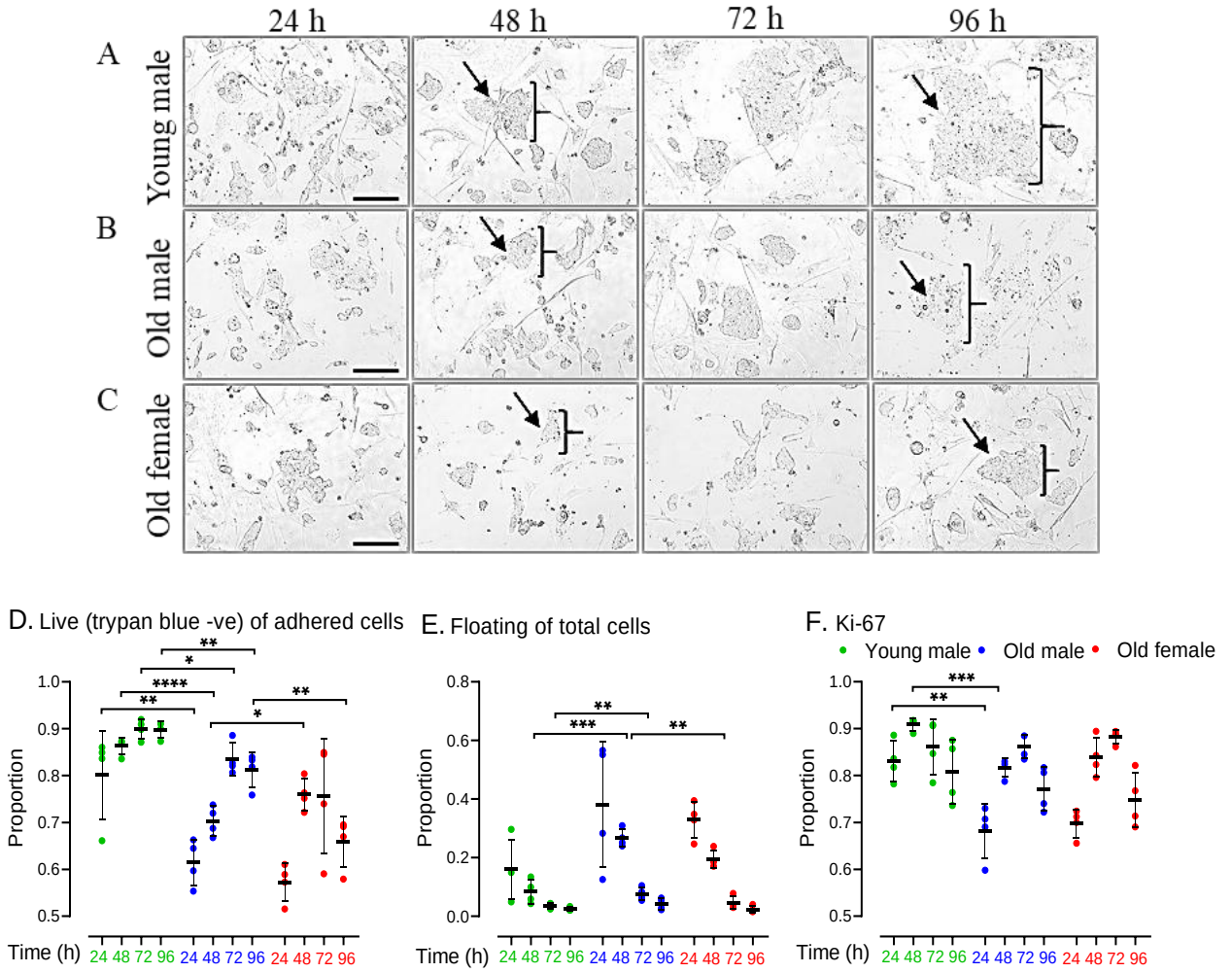

Figure 3. Cell proliferation and viability of undifferentiated Young male (7-8 weeks), Old (7-8 months) male, and female mESC lines. (A-C) Representative phase-contrast images of cell proliferation of Young male, Old male and Old female mESCs. After seeding, images were captured every 24 h over a period of 96 h. Images show that Young male **(A)** mESC lines have larger colonies with increased density of cells/ colony compared to Old male **(B)** mESC lines. Old Female mESC lines **(C)** present much smaller colony size and fewer mESC clones compared to Old Male mESC lines. Arrows and parenthesis point toward the colony size differences across the groups. Magnification = 100 µm. **(D, E)** Trypan negative (live) cells are presented as proportion of adherent (live and dead) cells, and floating cells as proportion of total (live, dead and floating) cells. **(D)** Old male mESC lines had fewer live adherent cells at all time points, and **(E)** increased proportion of floating cells at 48 h and 72 h, as compared to Young male mESC lines. **(F)** Protein expression of cell proliferation marker Ki67 over time presented as the proportion positive of total cells (stained and unstained). Ki67 expression was lower in Old vs Young mESC lines at 24 h (P=0.011) and 48 h (P=0.001). Data presented as means ± SDs based on n = 4 mESC lines per group. * P<0.01, ** P<0.001, *** P<0.0001, **** P<0.00001. Note: for **(D-F)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 4

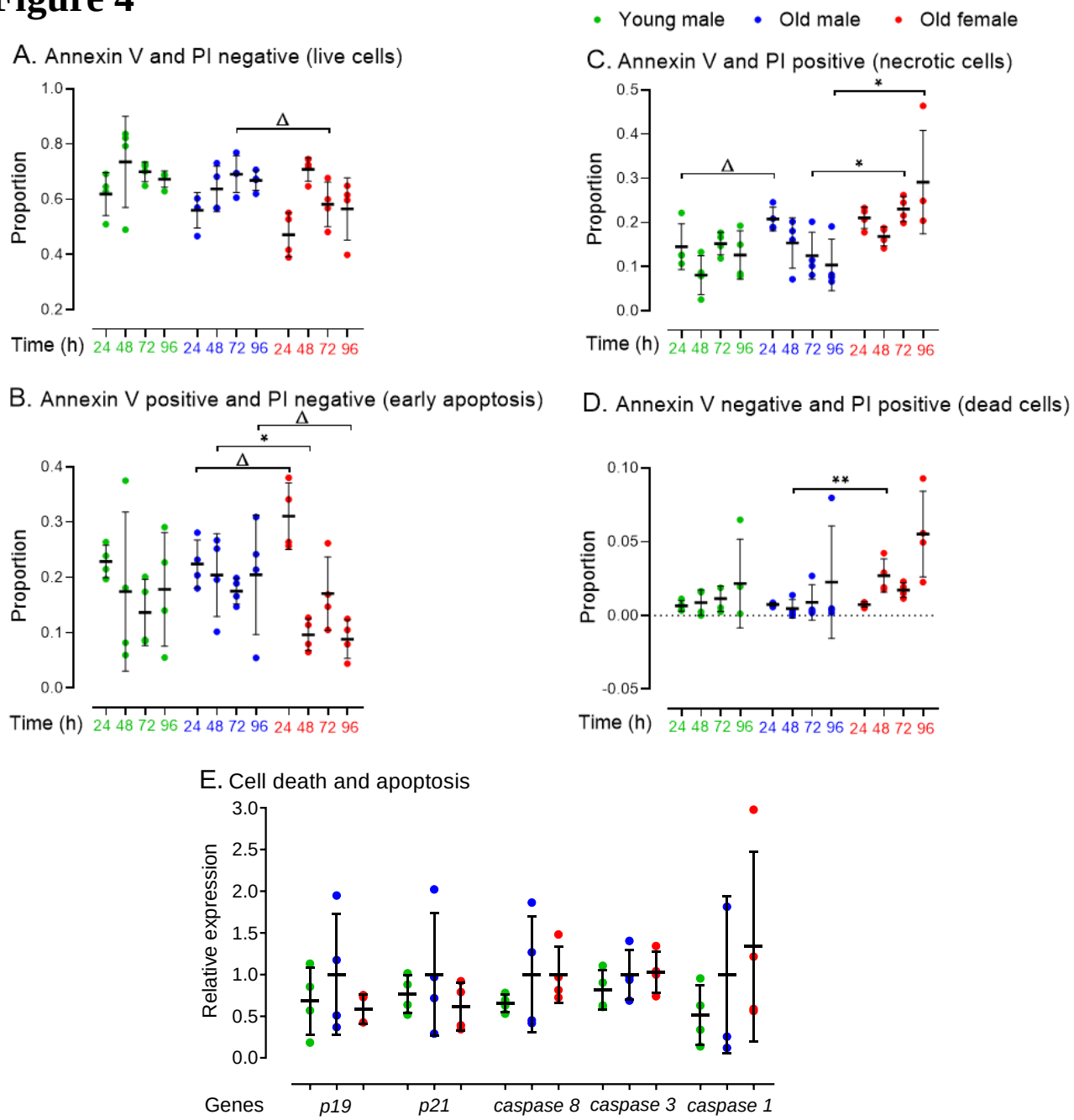

Figure 4. FACS analysis for detection of early apoptosis (Annexin V) and cell death (PI) in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male, and Old male vs Old female (7-8 months) mESC lines. mESCs were cultured in duplicate. **(A-D)** Data is presented as proportion of total cells. Compared to the Young group, Old mESCs presented (C) increased necrosis at non-significant trend at 24 h (P=0.075) although the (B) proportion of early apoptotic cells was unaffected by age and time. Sex differences were observed within the Old mESC group where Old Female mESC lines showed (B) increased proportion of early apoptotic cells at non-significant trend (P=0.059) at 24 h, which decreased at 48 h (P=0.055) and 96 h (P=0.085) also at non-significant trend. Old Female mESC lines also exhibited increased proportion of (C) necrotic cells at 72 h (P=0.035) and 96 h (P=0.035), and (D) dead cells at 48 h (P=0.01) compared to Old male mESC lines. (E) However, RT-qPCR analysis of cell apoptotic markers was unaffected by maternal age and sex. Data presented as means ± SDs based on n = 4 mESC lines per group, where ▵ = trending P<0.1, * = P<0.05 and ** = P<0.01. Note: for **(A-E)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.

Figure 5

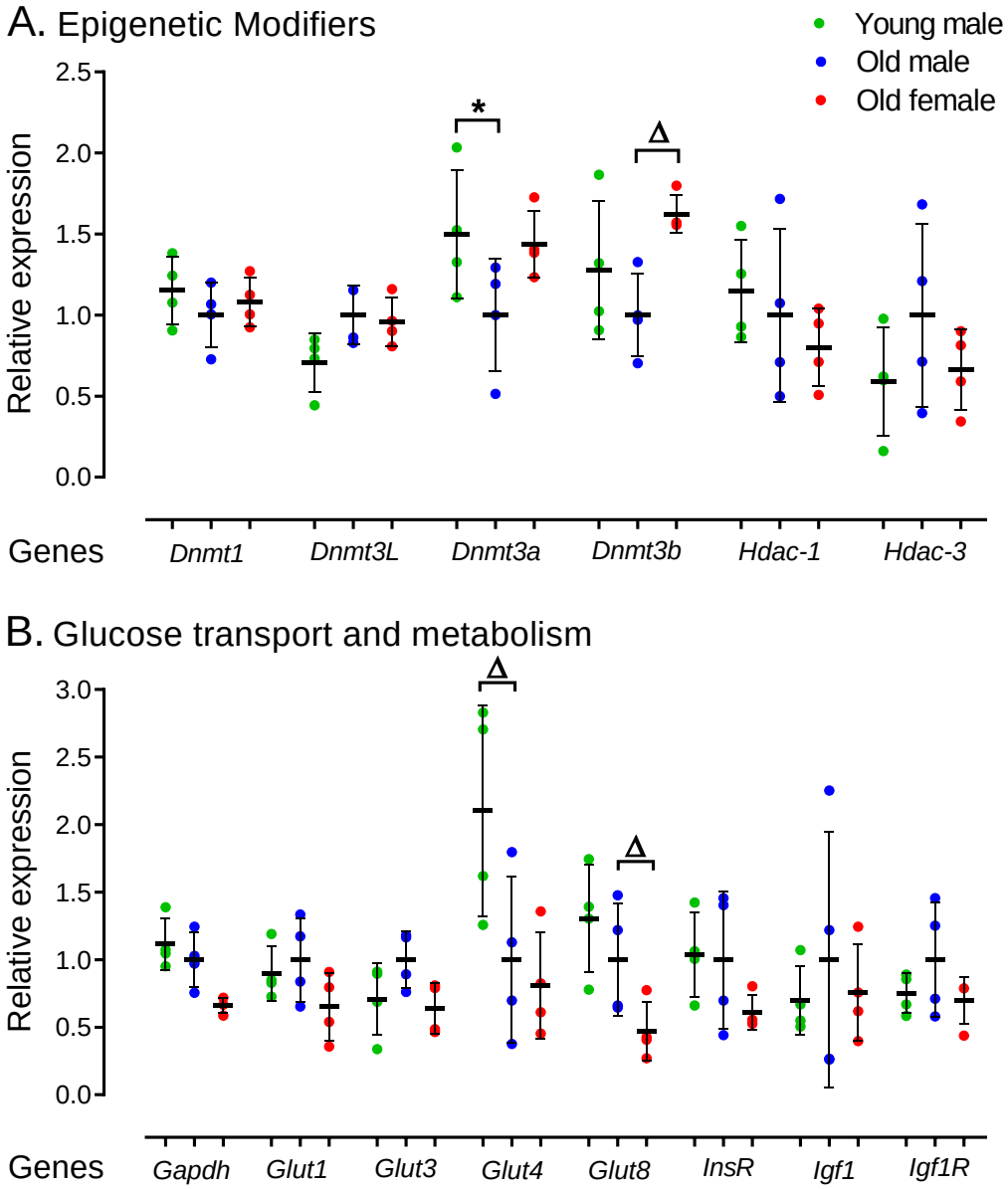

Figure 5. Relative gene expression for epigenetic modifiers and glucose transporters in undifferentiated Young (7-8 weeks) vs Old (7-8 months) male and Old male vs Old female (7-8 months) mESC lines. Real-time PCR analysis of DNA methyltransferases *Dnmt1*, *Dnmt3L*, *Dnmt3a* and *Dnmt3b*, histone modifiers *Hdac1* and *Hdac3*; glucose transporters *Glut1*, *Glut3*, *Glut4* and *Glut8* and metabolizers *Gapdh*, *InsR*, *Igf1* and *Igf1R*. Genes of interest were normalized to *Ywhaz* and *Rpl13a* within geNorm. (A, B) Old male mESC lines showed reduced expression of *Dnmt3a* (P=0.049) and *Glut4* at non-significant trend level (P=0.0685) compared to Young male mESC lines. Old Female mESC lines showed reduced expression of *Dnmt3b* at non-significant trend (P=0.078) and increased relative expression of *Gapdh* (P=0.029) and *Glut8* (P=0.055) at non-significant trend compared to Old Male mESC lines. Data presented as means ± SDs based on n = 4 mESC lines per group. * indicates P<0.05 and ▵ indicates trend P< 0.1. Note: for **(A,B)**, comparisons between Old Male and Young Male for ageing effects and between Old Male and Old Female for sex effects were normalised and analysed by t-test separately hence without post-hoc correction and placed in same chart for conciseness.