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EPIGENETICS

Female offspring sired by diet induced obese male mice display impaired blastocyst development with molecular alterations to their ovaries, oocytes and cumulus cells

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Abstract
Purpose To investigate the impacts that a paternal high fat diet (HFD) has on embryology, ovarian/cumulus cell gene expression and COC metabolism from female offspring, using a mouse model.
Methods Founder male mice were either fed a control diet (CD) or a HFD for 12 weeks. The HFD induced obesity but not diabetes, and founder males were then mated to normal weight CD fed female mice. Female offspring were maintained on a CD, super-ovulated, mated and the resultant zygotes were cultured to the blastocyst stage for embryo morphology, blastocyst cell number and apoptosis assessment. Ovaries and cumulus cells from offspring were collected for gene expression analysis of selected genes that maintain chromatin remodeling and endoplasmic reticulum (ER), metabolic and inflammatory homeostasis. Cumulus/oocyte complexes were also investigated for glucose uptake and lipid accumulation.
Results Female offspring sired by obese fathers produced embryos with delayed development and impaired quality, displayed increases in ovarian expression of *Glut1, Glut3* and *Glut4*, and an increase in cumulus cell expression of *Glut4*. Interestingly their COCs did take up more glucose, but did accumulate more lipid.
Conclusions A paternal HFD is associated with subfertility in female offspring despite the offspring being fed a CD and this subfertility is concomitant with ovarian/cumulus cell molecular alterations and increased lipid accumulation.

Keywords Embryo development · Blastocyst quality · Glucose transport · Ovarian gene expression · Cumulus cells

Capsule Although is has been previously demonstrated that paternal obesity can impede the fertility of female offspring, the development of embryos and the molecular charecterisation of reproductive material had not been investigated. Here we show that as a result of paternal obesity female offspring produce embryos with impaired development/quality, have alterations to the epxression of glucose tranpsorter genes in ovaries/cumulus cells and increased lipid uptake into cumulus-oocyte complexes.

Tod Fullston and Helana Shehadeh contributed equally to this work.

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Introduction

The global obesity epidemic is currently one of the most serious health concerns in the developed world and is an emergent concern in the developing world. More than one-third of men of reproductive age in the United States are classified as obese [1]. Paternal obesity not only impairs male fertility by impacting sperm count, motility and chromatin integrity [2–7] but also reduces subsequent embryo development, implantation rates, pregnancy success and live birth rates [8–12]. Interestingly metabolic syndrome, which includes obesity, in fathers is associated with polycystic ovary syndrome in daughters [13].

Additionally, animal models of paternal obesity demonstrate that males fed a high fat diet (HFD), inducing obesity with or without diabetes, generates daughters who have increased adiposity with metabolic disturbances such as

impaired insulin secretion, impaired glucose tolerance [14, 15] and molecular alterations in pancreatic islet cells [14] despite being fed a control diet (CD).

Furthermore, a model of paternal obesity which is the same as the one used here, previously demonstrated that female offspring had impaired oocytes. This was evident as a reduction in meiotic progression, increased oocyte arrest and altered mitochondrial function at 17 weeks of age, despite the offspring being fed a CD [16]. When mated to normal weight males, these females also give birth to F2 offspring who themselves have impaired metabolic and reproductive function [15, 16].

Overall, these studies suggest female offspring are obese as a result of paternal obesity induced developmental programming, resulting in detrimental effects to their metabolic and reproductive health. The perturbed reproductive health of female offspring sired by obese fathers was initially characterized by impaired oocyte maturation and impaired mitochondrial function at 17 weeks of age [16]. But the ability of these offspring to produce developmentally competent embryos and quality of their resultant embryos are not known for a much earlier age that predates any metabolic disturbances (i.e., 5 weeks of age). Furthermore, the molecular profile of ovaries and cumulus cells from these offspring at an optimal reproductive (i.e., 8 weeks of age) age has not been investigated previously.

Therefore, we hypothesize that the development and quality of resultant F2 embryos and cumulus/oocyte complexes (COCs) would be compromised and that gene expression would be altered in the ovaries and cumulus cells of females sired by fathers fed a HFD. We demonstrate that female offspring from fathers fed a HFD are subfertile, evident at 5–8 weeks of age as impaired embryo development/quality, increased lipid content in cumulus/oocyte complexes, increased expression of *Glut1*, *Glut3* and *Glut4* within ovaries and increased *Glut4* expression in cumulus cells. Interestingly, the subfertility phenotype described here occurs earlier in life (5–8 weeks) than the previously described reproductive pathologies (17 weeks) [16] and the molecular changes in offspring ovaries/cumulus cells differ to those described for female mice fed a HFD themselves [17, 18]. For the first time we demonstrate that a paternal HFD impairs the development of embryos produced from their daughters that predate any known metabolic disturbances, despite the daughters being fed a CD. Furthermore we demonstrate changes in gene expression occurs within the ovaries and cumulus cells from these animals, expanding the growing body of literature demonstrating that a paternal HFD impairs the reproductive health of female offspring.

Materials and methods

A flow diagram of experimental procedures can be seen in Fig. 1.

Mice and generation of offspring

Five week old male founder C57BL/6 mice (N=14) were randomly allocated to either a CD (N=6) (6 % fat, SF04-057; Specialty Feeds, Glen Forrest, Australia) or a nutrient matched HFD (N=8) (21 % fat, SF00-219; Specialty Feeds; Equivalent to Harlan Teklad TD88137). See Table 1 for a more detailed diet composition. C57BL/6 mice were selected as an obesity-prone strain [19], that we have previously used successfully to model obesity in the absence of overt diabetes [15, 16]. Males were housed individually and fed their respective diets ad libitum for 12 weeks with free access to water. At 13 weeks of age, 8 weeks post diet intervention, males were randomly mated with 6–8 week old, normal weight, CD fed and naturally cycling Swiss female mice. Swiss females were inspected for copulatory plugs daily and maintained on a CD throughout pregnancy and until weaning of their Swiss x C57BL/6 hybrid offspring. A paternal HFD did not alter litter size or offspring sex ratios (data not shown). Female (F1) offspring from CD or HFD fathers (two females sampled per litter) were maintained on a CD (N=20 and N=24 female offspring sired by CD and HFD males respectively, representing N=10 CD fathers and N=12 HFD). Body weight of all mice was measured weekly. Mice were housed at 18–24 °C in a 12:12 h light:dark cycle and animal usage and handling was approved by the University of Adelaide Ethics Committee (Ethics approval number M-61-09 18/05/09-30/06/12). All mice were maintained in accordance with NHMRC Australian code for the care and use of animals for scientific purposes [20].

Metabolic testing of founder males

Founder male mice underwent a glucose tolerance test (GTT) after 6 h of fasting at 12 weeks of age. An intra-peritoneal injection of 25 % glucose solution (Sigma-Aldrich, St Louis, USA) at 2 mg/g body weight was administered. Blood glucose concentration (mM) was measured from a tail blood sample using a glucometer (HemoCue; AB Angelholm, Sweden) and HemoCue® glucose microcuvettes 201 (HemoCue) at pre-injection 0 (baseline) and at 15, 30, 60 and 120 min post-injection. Data was collected for each individual animal and expressed as mean blood glucose concentration over time using area under the curve (AUC) analysis. Adipose tissues and organs were dissected and weighed post mortem.

Semen analysis of founder males

Sperm motility was assessed under x200 magnification; classified as motile or non-motile and expressed as a percentage of total sperm using 100 sperm per founder sample [21]. Sperm count was determined using a standard haemocytometer protocol [21].

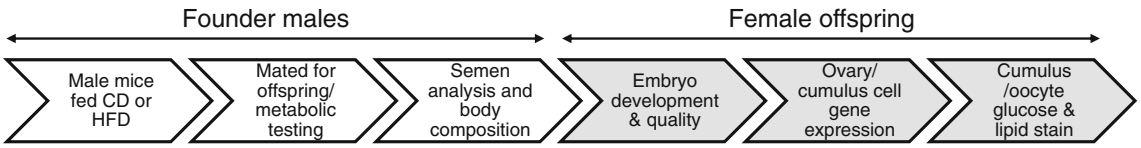

Fig. 1 Fathers were fed either a CD or HFD and female offspring were generated. Embryos from female offspring were cultured to assess embryo development and blastocyst quality. Ovaries and cumulus cells were obtained from female offspring for gene expression analysis. Cumulus/oocyte complexes were tested for glucose uptake and stained for lipid content

Intracellular ROS levels were assessed in a minimum of 20 motile sperm per sample by incubating sperm in a 5 μM concentration of di-chloro-fluorescein di-acetate; (DCFDA) for 15 min at 37 °C. Sperm were washed twice in PBS and using fluorescence microscopy and a photometer attachment, the relative fluorescence for each sample was expressed as mean fluorescence units [22, 23].

Embryo collection, culture and assessment

At 5–6 weeks of age, female offspring were super-ovulated via an intra-peritoneal injection of 5 IU PMSG (Folligon®; Intervet, Bendigo East, Australia) followed by 5 IU hCG (Pregnyl®; Organon, Australia) after 48 h and mated with a 9–10 week old CD fed C57BL/6 male overnight [24]. The presence of a copulatory plug confirmed mating and at 24 h post-hCG, cumulus enclosed zygotes were collected in G-MOPS (Vitrolife; AB Goteborg, Sweden) warmed to 37 °C. Zygotes were denuded in 0.5 mg/mL hyaluronidase for less than 2 min before culturing in groups of 10 in 20 μl drops [25] of G1 Plus (Vitrolife) for 48 h at 37 °C in 6 % CO_2, 5 % O_2 and 89 % N_2 . At 48 h, embryos were washed and cultured for a further 48 h in G2 Plus (Vitrolife). Embryo morphology and development were assessed using phase contrast microscope at 20 (day 2), 44 (day 3), 75 (day 4) and 92 (day 5) hours of culture. Embryo morphology was classified as: degenerate; 2–8 cells; morula; early, expanded, hatching or hatched blastocyst at each time point respectively.

Table 1 Control diet (CD) and high fat diet (HFD) compositions

Ingredients	CD (SF04-057)	HFD (SF00-219)
Sucrose (%)	34.1	34.1
Wheat starch (%)	30.5	15.5
Cellulose (%)	5.0	5.0
Casein (acid) (%)	19.5	19.5
Calcium carbonate (%)	1.7	1.7
Canola oil (%)	6.0	–
Clarified butter (%)	–	22.0
Methionine (%)	0.3	0.3
TD88137 Vitamins (%)	1.0	1.0
TD88137 Minerals (%)	0.14	0.14
Antioxidant (%)	0.004	0.004
Total Fat content	6 %	22 %

Blastocyst cell staining

Blastocysts were assessed for the number of inner cell mass (ICM) and trophectoderm (TE) cells according to a previously described protocol [26, 27]. Briefly, 0.5 % pronase was used to dissolve the zona pellucida, followed by a 10 min incubation at 4 °C in 10 % TNBS (Sigma-Aldrich). Blastocysts were then washed and incubated for 10 min at 37 °C in anti-DNP (Sigma-Aldrich) before incubation at 37 °C for 5–10 min in 10 μg/ml of propidium iodide (Sigma-Aldrich) in guinea pig serum (Sigma-Aldrich). Blastocysts were then transferred to 6 μg/ml bisbenzimide in ethanol overnight and washed in 100 % ethanol the following day. Blastocysts were mounted in glycerol on a microscope slide and examined using a fluorescent microscope under a UV filter.

The number of apoptotic cells per blastocyst was determined using a terminal deoxynucleotidyl transferase biotin-2′-deoxyuridine 5′-triphosphate nick-end labelling (TUNEL) technique as previously described [28]. Levels of DNA damage were measured using an in situ Cell Death Detection Kit (Roche; Manheim, Germany) and expressed as a proportion of total cells [28].

Ovary and cumulus cell RNA isolation

Ovaries and cumulus cells were obtained by post-mortem dissection and during embryo culture, respectively. Total RNA was isolated from ovaries and cumulus cells using RNeasy Micro kit (QIAGEN, Valencia, USA) according to the manufacturer's protocol. RNA concentration and purity were quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Samples from each female were processed individually.

Real-time reverse transcription PCR (qPCR)

Random hexamers (Invitrogen, Carlsbad, USA) and SuperScript III Reverse Transcriptase (Invitrogen) were used to reverse transcribe 1.0 μg of RNA from each ovary sample (N=6 per female offspring group) and all of the RNA

extracted from collected cumulus cell samples (N=8 per female offspring group). Primers were either designed using the NCBI Primer-BLAST and specifically confirmed with the UCSC Genome Bioinformatics database or were purchased as a Quantitect Primer assay (QIAGEN; Supplemental Table 1). Three technical replicates of qPCR were performed per sample using 2x Power SYBR Green Premix (Applied Biosystems, Foster City, USA). All primer pairs were verified to have sufficient amplification efficiency (i.e., >90 %) and to amplify a single specific product by agarose gel electrophoresis, melt curve analysis and direct Sanger sequencing of the qPCR product. The 96-well 7900 HT Fast Real-Time PCR System (Applied Biosystems) was used to thermocycle samples. All sample analysis was performed using the SDS 2.4 (Applied Biosystems) program. Results were normalized to the expression of a single reference gene, *18S* [29, 30], and then expressed as fold change in offspring sired by HFD fed fathers relative to offspring sired by CD fed fathers using the $\Delta\Delta C_t$ method (i.e., fold change=$2^{-\Delta\Delta Ct}$).

Collection of Cumulus/Oocyte Complexes (COCs)

8 week old female offspring were synchronised for ovulation by injection with 10 IU PMSG (Intervet). Ovaries were collected 46 h post PMSG injection and COCs were aspirated from antral follicles in GMOPS (Vitrolife) media [31].

Glucose uptake by blastocysts

Live COC's were incubated in 50 nl drops of GMOPS (Vitrolife) media with a 0.3 mM of 6-(N-(7-ntrobenz-2-oxa-1,3-diazol-4-yl)amino-6-deoxyglucose (6-NBDG) added (Molecular Probes, Eugene, USA). COCs were incubated for 60 min at 37 °C in 6%CO_2:5%O_2:89%N_2. Glucose uptake was then assessed by determining the amount of fluorescence in the COCs due to 6-NBDG using a photomultiplier with photometer attachment. The amount of fluorescence was determined to reflect glucose uptake as measured by quantitative microfluorescence. The distribution of glucose uptake to either the cumulus cells or oocyte was measured using confocal microscopy.

Lipid droplet staining and quantification

Prior to staining COCs were fixed with a 4 % paraformaldehyde (Sigma-Aldrich) in PBS solution overnight at 4 °C. COCs were stained with 1 μg/ml of the neutral lipid stain BODIPY 493/503 (Invitrogen) in PBS-PVP (1 mg/ml polyvinylpyrrolidone in PBS) for 1 h in the dark at room temperature. After staining, COCs were washed 2 times in PBS-PVP for 5 min. Images of each COC were captured by Leica SP5 spectral scanning confocal microscope using identical magnification and gain settings throughout all measurements taken. Using Analysis Pro software (Olympus), a rectangle was placed across the COC image and the average fluorescence intensity in each pixel column across the box was collated, a total of 971 intensities were taken per COC at 0.1875 μM intervals. The mean±SEM was calculated for all COCs per female offspring group and represented graphically as intensity of fluorescence over pixel widths and also grouped per cumulus cell (CC) and oocyte region.

Statistics

All values are reported as mean±SEM for all treatment groups including CD and HFD fathers; and female offspring from CD and HFD fed fathers. Statistical significance was determined for weight, body compositions, sperm parameters, differential staining (with paternal diet and day of experiment as covariates), TUNEL staining, glucose/lipid staining and normalized gene expression (i.e., raw ΔC_t values) using Student's t test. For embryo development data, chi-square tests were performed. All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., San Diego, CA) using a generalized linear model and LSD post-hoc test. For all analyses, $P \leq 0.05$ was defined as statistically significant.

Results

Paternal HFD elevates founder total body weight and increases adiposity without altering glucose tolerance

Males fed a HFD (N=7) had a significantly higher body mass ($P<0.05$), liver mass ($P<0.01$, Table 2), pancreas mass ($P<0.01$, Table 2) and a 77.3 % increase in the sum of adipose depots compared to CD males (N=6, P=0.001, Table 2). Organ weights and adiposity measured relative to total body weight demonstrated a 53 % increase in the sum of adipose depots ($P<0.001$), and decreases in testes ($P<0.01$) and seminal vesicle (P=0.05, Supplemental Table 3) weights in HFD fed males. However, glucose tolerance was not different between the two diet groups as determined by AUC analysis (CD: AUC 744.2±90.7 mM.min; and HFD 849.2± 113.4 mM.min; P=0.48). Overall this confirms that this HFD feeding regimen increases total body weight and adiposity in the absence of overt signs of diabetes.

Paternal HFD impairs founder sperm motility and quality

Males fed a HFD showed a significant reduction in the percentage of motile sperm ($P<0.01$), although there was no difference in sperm count (Supplemental Fig. 1). HFD fed males also displayed a significant increase in intracellular sperm

Table 2 Mass of adipose depots and organs from founder CD (N=6) and HFD (N=7) fed males

Parameter	CD male (grams)	HFD male (grams)	P value
Total body mass	28.2±0.87	32.7±0.73	<0.01
Peri-renal fat	0.20±0.02	0.27±0.08	NS [a]
Retroperitoneal fat	0.08±0.01	0.18±0.10	<0.05 [b]
Omental fat	0.31±0.04	0.52±0.14	<0.05 [b]
Dorsal fat	0.26±0.02	0.50±0.17	<0.01 [b]
Gonadal fat	0.89±0.08	1.63±0.29	<0.001 [b]
Sum of adipose depots	1.76±0.17	3.12±0.58	=0.001 [b]
Right testis	0.08±0.001	0.08±0.003	NS [a]
Left testis	0.08±0.002	0.08±0.001	NS [a]
Left epididymis	0.02±0.002	0.02±0.003	NS [a]
Seminal vesicles	0.31±0.012	0.31±0.013	NS [a]
Vastus lateralis	0.10±0.011	0.12±0.012	NS [a]
Soleus	0.01±0.004	0.01±0.004	NS [a]
Liver	1.08±0.036	1.42±0.095	<0.01 [c]
Pancreas	0.11±0.007	0.13±0.008	<0.05 [c]
Right kidney	0.19±0.004	0.20±0.007	NS [a]
Left kidney	0.19±0.008	0.19±0.007	NS [a]

[a] NS=not significant. [b] measure is significantly different as a proportion of total body mass (refer to Supplemental Table 2). [c] measure is not significantly different as a proportion of total body mass (refer to Supplemental Table 2)

ROS concentrations compared to CD fed males (P<0.001, Supplemental Fig. 1).

Female offspring produce embryos with delayed development

The number of embryos collected after superovulation did not differ between female offspring sired by either CD or HFD fed fathers (N=292 and N=330 presumed zygotes collected from offspring of CD and HFD fed fathers respectively). But female offspring sired by HFD fed fathers had a reduced proportion of embryos that fertilized and cleaved to the two-cell stage compared to female offspring sired by CD fathers (Table 3). The proportion of cleaved embryos that showed on-time development to the 8-cell stage either with or without the compaction of cells (day 3) was also reduced in female offspring sired by HFD fathers (P<0.0001, Table 3). This delay in embryo development was further evident on day 4 of culture with a decrease in the proportion of embryos that developed to the morula or blastocyst stages (P<0.05; Table 3). Interestingly if embryos were maintained in culture for a further 24 h until day 5, this delay in development dissipated (Table 3). A delay to embryonic development that is attenuated by the blastocyst stage is reminiscent of the embryo phenotype observed for embryos derived directly from HFD fed founders themselves [9, 11, 12, 32].

Table 3 Embryo development on days 2, 3, 4 and 5 of in vitro culture from female offspring sired by CD or HFD founder males

Parameter	CD father	HFD father	P value
Total presumed zygotes	292	330	NS
Day 2 cleavage	264 (90.4 %)	276 (83.6 %)	<0.05
Day 3 on time	192 (72.9 %)	134 (48.5 %)	<0.0001
Day 3 compacted	86 (32.4 %)	64 (23.1 %)	<0.05
Day 4 morula/blastocyst	237 (89.8 %)	229 (82.9 %)	<0.05
Day 4 total blastocyst	184 (69.6 %)	153 (55.4 %)	=0.001
Day 4 hatch blastocyst	32 (12.2 %)	22 (7.9 %)	NS
Day 5 total blastocyst	218 (82.7 %)	215 (77.8 %)	NS
Day 5 hatch blastocyst	136 (51.4 %)	121 (43.8 %)	NS

Percentage values from day 3 onwards are presented as a proportion of cleaved 2-cell stage embryos on day 2

NS not significant

Female offspring produce blastocysts with impaired quality

Although there was no difference in the total number of cells in blastocysts from females derived from a HFD fed father, there was a significant increase in TE cell number and consequently a reduced proportion of ICM cells (P<0.05, Fig. 2). TUNEL staining of blastocysts showed no difference in the proportion of apoptotic cells in the blastocysts from females sired by CD or HFD fathers (Fig. 2).

Altered ovarian gene expression in female offspring

The delayed development of embryos from F1 females sired by HFD fed fathers implicates molecular changes in their ovaries, which might be responsible for the observed delay in embryo development. The expression of the below genes have been previously shown to be altered in response to maternal diet induced obesity and increased adiposity were investigated [17, 18, 33–35]. In ovaries from females of HFD fathers relative to females of CD fathers, $\Delta\Delta C_t$ analysis showed that the expression of facilitated glucose transport genes, *Glut1; Glut3;* and *Glut4*, were significantly increased to 1.66±0.15; 1.44±0.18; and 1.79±0.29 fold, respectively (N=6 per treatment group, $P \leq 0.05$, Fig. 2 and supplemental Table 2). Other genes tested that are involved in endoplasmic reticulum (ER) stress (*Atf4; Atf6; Hspa5*; and *Xbp1*), metabolic sensing (*Sirt3, Sirt4,* and *Sirt5*), inflammatory stress (*Olr1* and *Scarb1*) and chromatin remodeling (*Smarca1, Smarca2, Smarca4, Smarca5,* and *Atrx*) did not show significant differences in expression between the two groups of female offspring (Supplemental Table 2). The alteration to the expression of glucose transporter genes was not concomitant with any changes to fasted serum concentrations of insulin (CD father 0.12±0.02 ng/ml; HFD father 0.13±0.01 ng/ml; P=0.43).

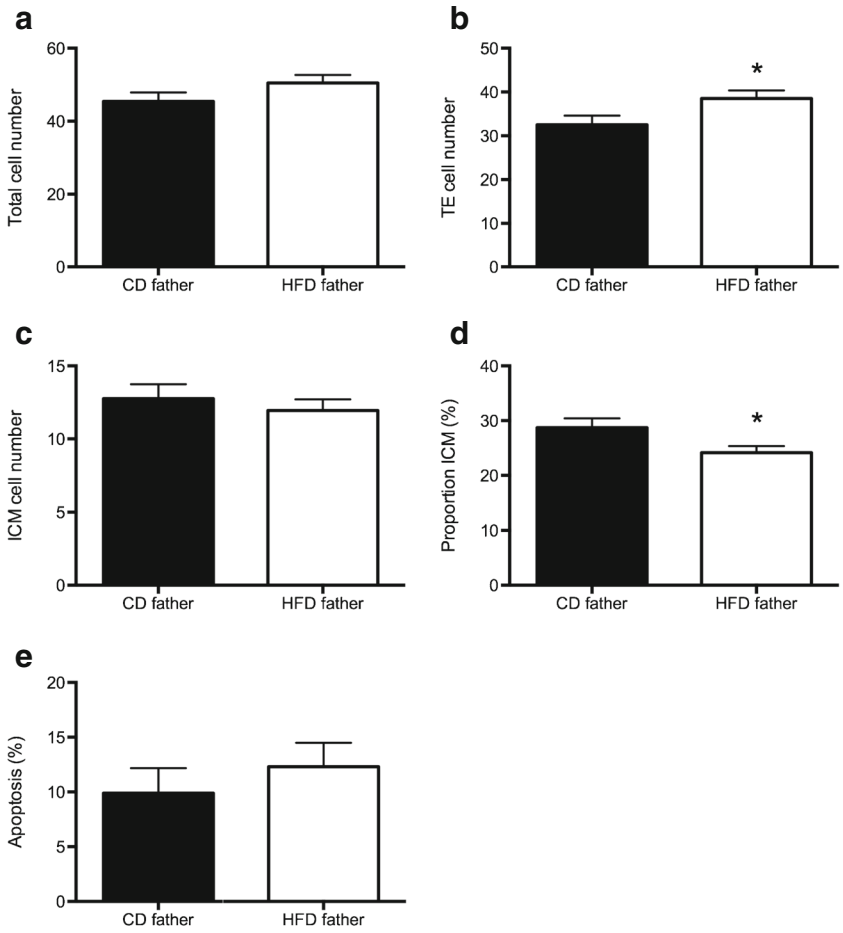

Fig. 2 Blastocyst cell number and differentiation from 5 to 6 week old females sired by either a CD (N=55) or HFD (N=73) fed father: (**a**) Total cell number, (**b**) trophectoderm (TE) cell number, (**c**) inner cell mass (ICM) cell number, (**d**) proportion of ICM cells and (**e**) proportion of apoptotic cells in blastocysts from female offspring sired by CD and HFD fed fathers. Values are expressed as mean±SEM (*P≤0.05)

Altered cumulus cell gene expression in female offspring

Glut1, *Glut3* and *Glut4* were selected for further gene expression analysis in cumulus cells from offspring sired by HFD fed males based on their increased expression in the whole ovary. *Glut4* expression was increased to 3.49±0.53 fold in cumulus cells from female offspring sired by HFD fed fathers compared to offspring sired by CD fed fathers (P≤0.05, Fig. 3). However, *Glut1* expression was not different in cumulus cells from female offspring sired by HFD fed fathers compared to CD fed fathers and *Glut3* expression was below the detectable limits of the qPCR assay.

Glucose uptake is unchanged in Cumulus/Oocyte Complexes (COCs) from female offspring

Given the increased expression of the glucose transporter *Glut4* in cumulus cells, the uptake of glucose by COCs derived from female offspring sired by CD (N=18) or HFD (N=23) fathers was investigated. No significant differences in glucose uptake were observed between the COCs from female offspring sired by either CD or HFD fathers, despite the numerical increase seen in COCs from females sired by HFD fed males (Fig. 4)

Increased lipid droplet content in COCs derived from female offspring

It has been previously demonstrated that daughters sired by HFD fed fathers have increased adiposity [15] and that diet induced female obesity can increase lipid accumulation in COCs [18], thus we examined lipid accumulation in COCs from female offspring. Lipid droplet accumulation was increased in COCs derived from female offspring born to HFD fed males (N=23), compared to COCs from females born to CD fed males (N=20) in both cumulus cells and oocytes (Fig. 5).

Discussion

This study adds to a growing body of literature linking paternal obesity to impaired offspring health [14–16]. We demonstrate that a peri-conceptional paternal HFD is associated with the impairment of their daughters' reproductive health,

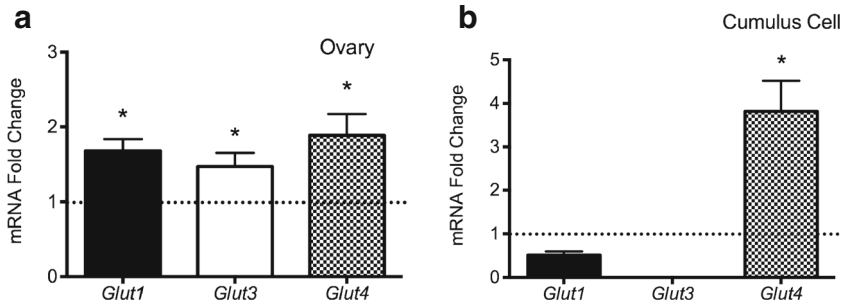

Fig. 3 Fold change in expression of (**a**) ovary (N=6) and (**b**) cumulus (N=8) mRNA in 8 week old offspring sired by HFD fed fathers. Values are normalized to *18S* expression and graphed relative to offspring sired by CD fed father (i.e., CD set to 1.0 at the dotted line, N=6 and N=8 offspring from CD fed fathers for ovary and cumulus cell expression respectively). Values are expressed as mean±SEM (*$P \leq 0.05$)

demonstrated by lipid accumulation in their cumulus/oocyte complexes (COCs), delayed embryo development, impaired blastocyst quality and increases in the expression of glucose transporters in both the ovary and cumulus cells. The subfertility phenotype documented here, at 5–8 weeks of age, is earlier in life than the reproductive defects previously reported at 17 weeks of age using the same paternal HFD model [16]. Moreover this subfertility occurs concomitantly with molecular changes in offspring ovaries/cumulus cells that differ to those reported for female mice directly fed a HFD, but with similar lipid accumulation in COCs [17, 18].

The model of paternal diet induced obesity used here recapitulated that sperm from males fed a HFD had reduced motility and increased levels of intracellular ROS, compared to sperm from CD fed males. This is consistent with previous studies in similar models that demonstrate that not only does HFD feeding result in an obese phenotype [9, 11, 12, 14–16, 32, 36–38] but also correlates with impaired sperm quality [15, 16, 36–38]. Although testosterone concentrations were not measured in these HFD fed males, we speculate that their observed subfertility may be associated with lowered testosterone concentrations, as previously demonstrated [36, 37].

Paternal diet induced obesity and its impact on the health of female offspring has been investigated previously with regards to both metabolic and reproductive health. Metabolic disturbances in female offspring sired by males fed a HFD have been shown to develop; both impaired glucose tolerance and insulin sensitivity from 8 to 14 weeks of age respectively without changes to fasted serum insulin concentrations [15], as also observed for this cohort of female offspring. Furthermore, the paternal transmission of subfertility to the next generation has been demonstrated previously, albeit later

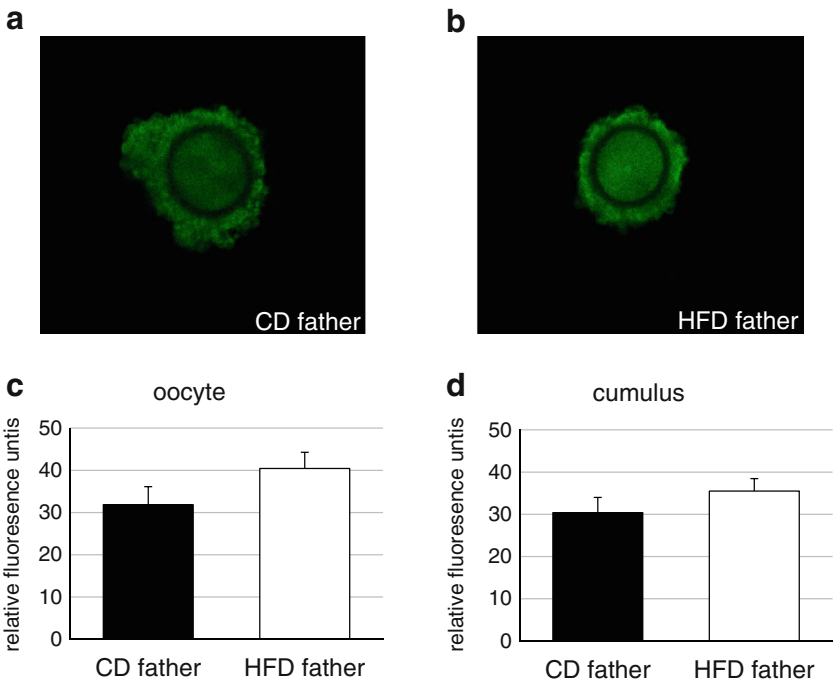

Fig. 4 6-NBDG glucose stain intensity in COCs from 8 week old offspring. Representative images are shown for COCs from offspring sired by (**a**) CD and (**b**) HFD fed fathers. Mean stain intensity for (**c**) cumulus cells or (**d**) oocytes sired by CD (N=18) and HFD fed fathers (N=23). Values are expressed a mean± SEM

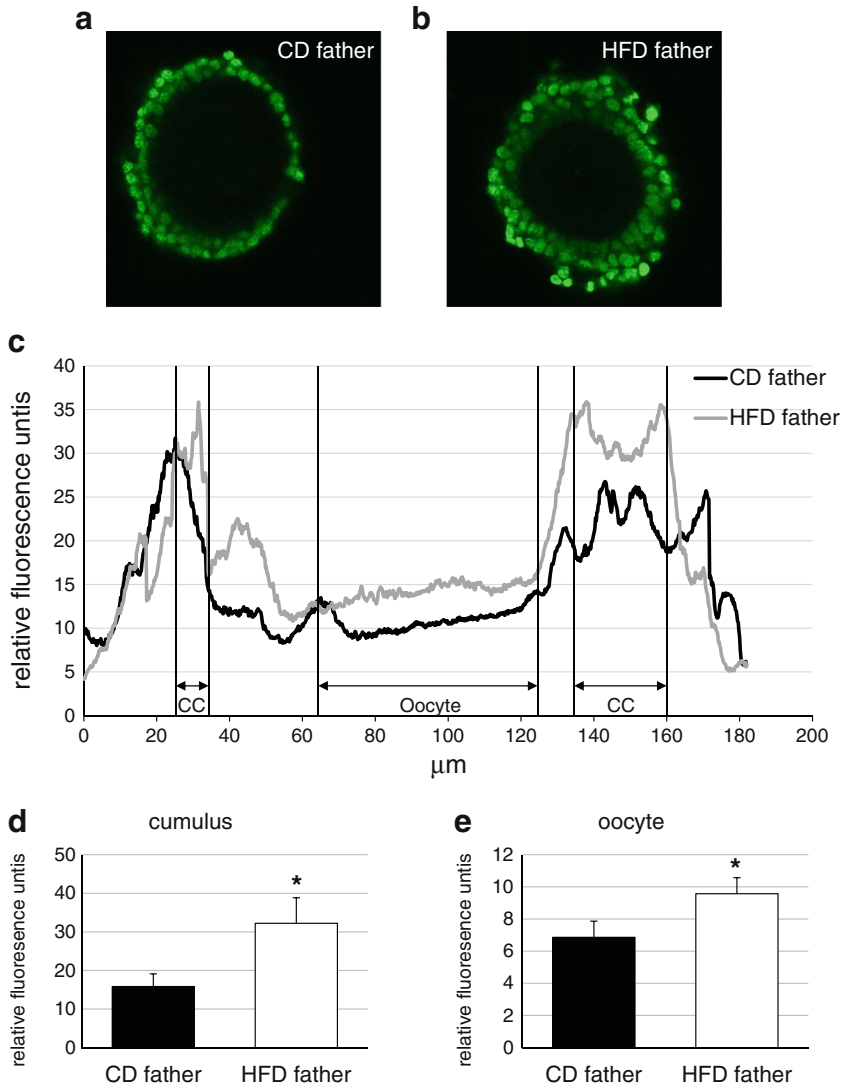

Fig. 5 BODIPY staining intensity in COCs from 8 week old offspring. Representative images are shown for COCs from offspring sired by (**a**) CD and (**b**) HFD fed fathers. (**c**) Mean cross sectional stain intensity for COCs from offspring sired by CD (*black line*; *N*=20) and HFD (*grey line*; *N*=23) fed fathers, and mean stain intensity for cross sections demarked in panel c as (**d**) cumulus cell (CC) or (**e**) oocyte. Values are expressed a mean± SEM (**P*<0.05)

in life (17 weeks of age); whereby oocytes from female offspring sired by obese fathers had reduced meiotic competence and impaired mitochondrial function [16]. This current study extended these findings to determine that there was a negative impact on fertilization and cleavage to the two-cell stage and that embryo development was delayed at key milestones of compaction and blastulation, apparent much earlier in life (5–6 weeks of age). Interestingly, when embryos from daughters of HFD fathers were cultured for an additional day (to day 5), they exhibited developmental 'catch up', demonstrating a phenotype of delay in development rather than a failure to develop to the blastocyst stage.

Of the embryos that developed to blastocysts, embryo quality was measured by the use of markers of the first differentiation event in the embryo, the differentiation of the ICM from the TE. Although total cell number (mitosis) was not altered, there was an increas to the proportion of TE cells with a corresponding decrease in the proportion of ICM cells in the blastocysts produced by offspring from a HFD father, compared to that from a CD father. This alteration to early cellular partitioning potentially indicates reduced embryo quality. Previous studies demonstrate that an alteration to the number of both TE and ICM cells in the blastocyst are associated with reduced implantation rates [9, 39–41], although implantation is presumably more sensitive to TE cell number. Overall, embryos from female offspring sired by obese fathers displayed delayed development and impaired quality despite the offspring themselves being fed a control diet, mimicking the developmental profile of embryos sired directly by HFD males themselves [9, 12, 32, 42].

We further examined the ovaries from the female offspring to determine if the delayed development we observed may be explained by altered gene expression. As offspring sired by obese fathers have previously been shown to have increased adiposity [15, 16] a selection of genes that have been shown to be altered by diet induced obesity (i.e., HFD fed) were

investigated. Thus genes involved in metabolic stress, ER stress and inflammatory stress responses or glucose transport and chromatin remodeling were targeted in ovarian tissue. Metabolic markers, in particular glucose transport target genes (*Glut1, Glut3* and *Glut4*), were found to have increased ovarian expression in offspring sired by HFD fed males compared to that sired by CD fed males. This highlights that the altered gene expression in ovaries of the female offspring born to HFD males is limited to a subset of genes that are altered by direct HFD feeding to female mice.

Glucose is an essential energy source in the ovary, required for maintaining ovarian metabolism. Glucose cannot permeate the plasma membrane and several transporters that have relatively different functions mediate glucose uptake. *Glut1* is a ubiquitous transporter [43], *Glut3* is a high affinity transporter [43] and *Glut4* is an insulin-responsive glucose transporter [34, 44–46]. Our results show that *Glut1, Glut3* and *Glut4* have increased ovarian expression in daughters of HFD fed fathers, indicating a potential increase in glucose transport in ovarian cells. This may impair oocyte quality as previous studies demonstrate high glucose environments (e.g., diabetic mouse models) impair oocyte quality and development, presumably by altering the glucose levels in the follicular environment [47–49]. Moreover, elevated concentrations of glucose in the uterus are associated with poor oocyte developmental competence in vitro [50].

The ovary is a heterogeneous organ and therefore the expression of *Glut1*, *Glut3* and *Glut4* were investigated at a cell-specific level, *i.e.,* in cumulus cells. Glucose transporters have previously been shown to be expressed in cumulus cells and function as a mediator for glucose uptake and further transfer glucose to the oocyte [51]. Interestingly, only *Glut4* expression was found to be significantly elevated in cumulus cells from offspring sired by a HFD fed father, which may have implications for cumulus cell mediated oocyte-supporting role via glucose transport [51, 52]. As *Glut4* is an insulin-responsive glucose transporter [34, 44–46], the observed increase in expression suggests elevated insulin concentrations, although not insulin was not elevated in the sera of these animals. Previous reports have shown that obese women have increased insulin concentrations in their follicular fluid compared with normal weight women [34, 53, 54], however *Glut4* expression in their cumulus cells was not increased [34] and follicular insulin was not measured in this study. Furthermore, *Glut4* expression has been reported in cumulus cells from women seeking infertility treatment, but its abundance was not correlated with oocyte quality and its relationship to blastocyst development and live birth could not be established [55]. This suggests that increased *Glut4* expression in cumulus cells is specific to offspring sired by a HFD fed father and might result from their programmed obesity (i.e., not HFD diet induced), or is perhaps a species specific phenomenon.

Interestingly the increased expression of *Glut4* in both ovaries and cumulus cells from female offspring born to HFD fathers did not result in increased glucose uptake by COCs, but did result in increased lipid accumulation; consistent with diet induced female obesity studies [18]. A larger sample size of COCs for glucose uptake might lend sufficient power to detect elevated glucose (~+25 %), or perhaps the use of heterozygous female offspring strain (Swiss x C57BL/6) may have resulted in too much variation for a significant difference to be detected. Perhaps the transcription of *Glut4* mRNA in the cumulus cells in COCs at this stage preempts any effect on the translation of the protein and thus the impact on glucose uptake is not observed yet, or any increased glucose uptake is shared between the cumulus cells and the oocyte. Indeed it remains possible that the increased *Glut4* mRNA expression does not impact upon glucose transport at all, clearly further investigation is warranted.

This study demonstrates delayed development and impaired quality of embryos in female offspring sired by a HFD fed male, concomitant with alterations to ovarian and cumulus cell gene expression and increased lipid accumulation. There are three potential explanations for the observed impaired reproductive health in these female offspring. Firstly, there may be a paternal transmission of impaired fertility due to epigenetic modifications in sperm as a result of their father's consumption of a HFD [15, 56–58]. Secondly, a paternal HFD may have initiated metabolic disturbances in offspring (impaired glucose tolerance and insulin sensitivity), which may cause the subfertility reported here. Although it must be noted that the female offspring examined for embryology in this study were 5–6 weeks of age and previous investigations using the same paternal HFD model document metabolic disturbances from 8 weeks of age [15]. Thirdly, there may be a direct effect on the micro-environment of the ovarian follicle in female offspring, which may contribute to the observed increased lipid accumulation in COCs, delayed embryo development and impaired blastocyst quality [48, 59].

In summary, these results illustrate a paternal influence on the outcome of their daughters' fertility and have implications for idiopathic subfertility in a clinical setting. Continued evidence of detrimental intergenerational effects due to paternal obesity in animal models warrant studies in human cohorts.

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Conflict of interest T.F., H.S., L.Y.S., W.X.K., L.L.W., R.L.R., N.O.M. and M.L. declare that they have nothing to disclose.

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