When two obese parents are worse than one! Impacts on embryo and fetal development

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1School of Paediatrics and Reproductive Health, Robinson Research Institute, University of Adelaide, South Australia, Australia; 2Freemasons Centre for Men’s Health, University of Adelaide, South Australia, Australia; 3Repromed, Dulwich, South Australia, Australia; and 4Monash In Vitro Fertilisation Group, Richmond, Australia

Submitted 12 May 2015; accepted in final form 14 July 2015

McPherson NO, Bell VG, Zander-Fox DL, Fullston T, Wu LL, Robker RL, Lane M. When two obese parents are worse than one! Impacts on embryo and fetal development. Am J Physiol Endocrinol Metab 309: E568–E581, 2015. First published July 21, 2015; doi:10.1152/ajpendo.00230.2015.—The prevalence of overweight and obesity in reproductive-age adults is increasing worldwide. While the effects of either paternal or maternal obesity on gamete health and subsequent fertility and pregnancy have been reported independently, the combination of having both parents overweight/obese on fecundity and offspring health has received minimal attention. Using a 2 × 2 study design in rodents we established the relative contributions of paternal and maternal obesity on fetal and embryo development and whether combined paternal and maternal obesity had an additive effect. Here, we show that parental obesity reduces fetal and placental weights without altering pregnancy establishment and is not dependent on an in utero exposure to a high-fat diet. Interestingly combined parental obesity seemed to accumulate both the negative influences of paternal and maternal obesity had alone on embryo and fetal health rather than an amplification, manifested as reduced embryo developmental competency, reduced blastocyst cell numbers, impaired mitochondrial function, and alterations to active and repressive embryonic chromatin marks, resulting in aberrant placental gene expression and reduced fetal liver mtDNA copy numbers. Further understanding both the maternal cytoplasmic and paternal genetic interactions during this early developmental time frame will be vital for understanding how developmental programming is regulated and for the proposition of interventions to mitigate their effects.

The prevalence of overweight and obesity in reproductive-age adults is increasing worldwide (62). For example, in America it is estimated that 70.9% of males and 61.9% of women are classified as overweight/obese (62). The comorbidities associated with obesity have been well defined; however, until recently the impact on pregnancy and fetal development has been less recognized. Although the effects of either paternal or maternal obesity on gamete health and subsequent fertility and pregnancy have been reported independently, the combination of having both parents overweight/obese on fecundity and offspring health has received minimal attention. This information is essential, as the percentage of couples of reproductive age with both partners overweight/obese is increasing and is the predominant situation in many countries (62).

To date, there have only been three studies that have assessed the combined effects of maternal and paternal obesity on pregnancy and fetal health, two in human-assisted reproductive technology (ART) cohorts and one using a rodent high-fat diet model (41, 67, 76). These studies found no effect of maternal and paternal obesity on pregnancy establishment (41, 67, 76); however, when offspring health was investigated in the rodent model, insulin resistance and liver steatosis were greatest in offspring where both parents had been feed a high-fat diet prior to and during gestation compared with just one parent (67).

The amplified effect seen with two obese parents in that study was likely compounded as a result of the individual effect obesity has on each gamete (45). For example, maternal obesity is associated with numerous markers of reduced oocyte quality, including impaired maturation (17, 35), increased lipid content (86, 87), cellular lipotoxicity (87), alterations to mitochondria including ultrastructure (30), membrane potential (32), and mitochondrial DNA (mtDNA) content (32, 88), as well as increased reactive oxygen species and depleted glutathione (indicative of oxidative stress) (32, 52) and alterations to DNA methylation patterns of key metabolic and imprinting genes in oocytes (29), resulting from reduced DNA methyltransferase levels (28, 29). In males, paternal obesity increases sperm oxidative stress and DNA damage (3, 38, 69, 81) and alters sperm microRNA content (23, 55), lipid content (20), protein composition (39, 51, 70, 77), and active and repressive chromatin state (68) and global DNA hypomethylation (23). Importantly, since both maternal and paternal obesity have been independently correlated with programming perturbed metabolic health in offspring, the potential compounding effect of two obese parents must be examined. Furthermore, the extent to which altered offspring phenotypes arise from changes during early embryo development vs. via altered placental growth and function later in gestation remains to be determined.

Therefore, the aims of this study were to determine 1) whether key embryonic milestones are related to impaired fetal health and what influence exposure to the reproductive tract in high-fat diet-fed females may have on outcomes, 2) whether previously reported sperm and oocyte changes resulting from high-fat diet exposure persists in the early embryo and how this relates with fetal and placental function, and 3) whether combined maternal and paternal high-fat diet exposure has an additive effect.
MATERIALS AND METHODS

Animal Model and Diets

Five-week-old male (n = 26) and female (n = 162) C57BL6 mice were randomly allocated to either a control diet (CD, 6% fat, SF04-057; Specialty Feeds, Perth, Australia) or a nutrient-matched high-fat diet (HFD, 21% fat, SF00-219; Specialty Feeds). Males were fed their respective diets for 9 wk (2 complete rounds of spermatogenesis), which is known to perturb sperm function (3), whereas females were fed their diets for 6 wk, which has previously been shown to impair oocyte mitochondrial function and health (32). Both time periods were sufficient to increase adiposity but not impair glucose homeostasis and therefore did not introduce additional glycaemic or hyperinsulimic influences (64). Males were individually housed, while females were housed in groups of five. Mice had free access to food and water and were maintained on a 12:12-h light-dark cycle.

Ethical Approval

All animals were maintained according to the National Health and Medical Research Council Guidelines for the care and use of animals. The Animal Ethics Committee of the University of Adelaide approved the use and care of all animals used in the study.

Analysis of Body Composition

Individual animal body weights were recorded weekly. Body composition was assessed by dissection at post mortem for adipose depots (omentum, peritoneal, perirenal, and gonadal) and organ weights (liver, pancreas, kidneys, testes, and ovaries).

Glucose Tolerance Testing

Animals were fasted for 6 h and administered an intraperitoneal injection (ip) of 2 mg/g ip 25% glucose (63) (n = 8 animals per sex per diet treatment). Blood glucose concentration (mmol/l) in tail blood was measured using Hemocue Glucose cuvets 201+ (Hemocue no. 120717, Angelholm, Sweden) preinjection, for a baseline reading (0 min, fasting glucose reading), and at 15, 30, 60, and 120 min after glucose bolus. Glucose tolerance of individual animals was determined by glucose clearance as measured by area under the curve (AUC).

Media, Reagents, and Chemicals

All reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. MOPS-G1 medium was used for embryo handling, G-IVF for in vitro fertilization, and G1.3 and G2.3 media for embryo culture (Vitrolife, Sweden). All media were supplemented with 10% human serum albumin (Vitrolife) unless otherwise stated. All reagents and plasticware were tested for embryo compatibility by using a 1-cell mouse assay (25).

Natural Mating and Pregnancy Outcomes

After 6 wk of diet exposure, female mice were randomly paired with either one CD or one HFD male for a period of 4 nights. After the presence of a copulatory plug, females were removed from the male cage and returned to group housing. On day 18 of pregnancy, uterine contents were examined for total number of implantation sites and litter size. Fetal and placental weights and lengths were recorded.

In Vivo Embryo Development

After 6 wk of diet exposure, female mice were injected with 10 IU PMSG ip and 10 IU hCG ip administered 48 h apart and were randomly paired with a CD or HFD male overnight. After the presence of a copulatory plug, embryos were flushed from either the oviduct 44 h post-hCG injection or from the uterus 90 h post-hCG injection with MOPS-G1. Embryo morphology and subsequent development were assessed using a phase contrast microscope, with embryos expected to be at two-cell or greater 44 h post-hCG and expanded blastocyst at 90 h post-hCG.

In Vitro Zygote Collection, Culture, and Morphological Assessment

After 6 wk of diet exposure, CD and HFD females were superovulated by injection of 10 IU PMSG ip and 10 IU hCG ip administered 48 h apart and were randomly paired with a CD or HFD male overnight. After the presence of a copulatory plug, zygotes were collected into MOPS-G1 and denuded in 0.5 mg/ml hyaluronidase before culture in G1 medium for 48 h, followed by a further 8 h of culture in medium G2 until the blastocyst stage at 37°C in 6%CO2-5%O2-89%N2 (60). Embryo morphology and subsequent on-time development were assessed using a phase contrast microscope at 26 h (≥2 cells), 43 h (8 cells/morula), 74 h (early-expanding blastocyst), and 91 h (expanded-hatching blastocyst) (60).

Differential Staining of Blastocysts

Differential nuclear staining was used to quantify the number of inner cell mass (ICM) and trophectoderm (TE) cells for in vivo- and in vitro-derived blastocysts at 90 ± 1 h post-hCG, as described previously (26). The number of ICM and TE cells [added together for total cell number (TCN)] were visualized under ultraviolet filter on the fluorescence microscope, with TE cells appearing pink and ICM appearing blue.

Embryo Transfers and Pregnancy Outcomes

To restrict the effects of maternal obesity to pre-conception and preimplantation stage development, embryos were transferred to normal-weight surrogates for gestation. Blastocysts were vitrified after 91 h of culture in High Security Vitrification Straws (Cryo Bio Systems, Paris, France), and embryo warming was performed as previously described (89). Warmed blastocysts were transferred into day 3.5 pseudo-pregnant Swiss female mice, and five morphologically similar blastocysts were randomly allocated to each contralateral uterine horn (60). On day 18 of pregnancy, uterine contents were examined for total number of implantation sites, resorption sites, and viable fetuses. Fetal and placental weights and lengths were recorded.

Immunocytochemistry Epiblast Staining (Nanog and Oct4)

For Nanog and Oct4 staining, blastocysts were cultured for a further 24 h (115-h culture) in the above-mentioned G2 conditions to allow cell lineage-specific localization of proteins to the epiblast and stained using previously published methods (12). ICM is determined by nuclei stained positive for Oct4, and epiblast cell number for nuclei stained positive for both Oct4 and Nanog.

Mitotracker Green FM Staining in Two-Cell Embryos

Two-cell embryos collected from mated females 44 h post-hCG injection were washed in MOPS-G1 before fixation in 4% paraformaldehyde for 45 min at room temperature and stored in PBS-PVP. Embryos were then incubated for 1 h in 100 μM Mitotracker Green (M-7514, Life Technologies) MOPS-G1 and washed through MOPS-G1 minus HSA. Embryos were imaged under epifluorescent microscopy, and the relative level of Mitotracker Green in each was determined by pixel intensity in Adobe Photoshop CS3 minus the background reading. Abundance of Mitotracker Green was expressed relative to embryos from CD M × CD F matings.

Immunocytochemistry Chromatin Staining (H3K4me3, H4K27me3) in Two-Cell Embryos

Chromatin staining of two-cell embryos was performed by immunocytochemistry. Briefly, two-cell embryos were collected 44 h post-hCG injection into MOPS-G1 before fixation in 4% paraformaldehyde.
placenta sample (Invitrogen, Carlsbad, CA) and SuperScript III Reverse Transcriptase. Real-Time Reverse Transcription PCR (qPCR) in Placentas

Quantification of mtDNA Copy Number in In Vivo Fetal Liver

DNA was extracted from frozen fetal liver tissue from fetuses from the matched position in contralateral uterine horns per pregnant mother. Briefly, each dissected tissue was added to 250 μl of digestion buffer (50 mM Tris-HCl, 20 mM EDTA, 120 mM NaCl, 1% SDS, pH 8). Tissues were homogenized by disposable tissue grinder pestle followed by the addition of 40 μl of proteinase K (10 mg/ml in sterile water) and incubated for 12 h at 37°C with gentle shaking. Ammonium acetate (250 μl 4 M, pH 7.5) was then added followed by gentle mixing and centrifugation at 14,000 rpm for 10 min. DNA was precipitated from the supernatant by adding 800 μl of absolute ethanol and overnight incubation at −20°C. DNA was pelleted by high-speed centrifugation followed by resinsing the pellet twice with 1 ml of 70% ethanol. After air drying, the pellet was resuspended in 100 μl of PCR-grade water. DNA extracted from fetal liver tissues was used to estimate average mtDNA copy number/cell as described (1). The primers for amplification of the mitochondrial gene (12S rRNA) were 5′-CGT TAG GTC AAG TAG TAG CC-3′ and 5′-CCA GAC ACA CTT TCC AGT ATG-3′. The primers for amplification of the nuclear gene (β-actin) were 5′-GGAAAAGAGCCTCAGGGCAT-3′ and 5′-CTGCCTGACGGCCAGG-3′. Quantitative PCR of mtDNA and nuclear DNA was performed simultaneously in each sample in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) and a Rotor-Gene 6000 (Corbett) real-time rotary analyzer. The Ct value for β-actin was subtracted from that for 12S rRNA to give the ΔCt value. mtDNA copy number per nuclear genome (two actin gene copies) is calculated as 2 × 2ΔCt.

Real-Time Reverse Transcription PCR (qPCR) in Placentas

Total RNA was extracted from placental samples using Tri Reagent, per the manufacturer’s protocol (Sigma). Random hexamers (Invitrogen, Carlsbad, CA) and SuperScript III Reverse Transcriptase (Invitrogen) were used to reverse transcribe 5.0 μg of RNA from each placenta sample (n = 10–12 per group; 2 fetus from contralateral uterine horns per mother). qPCR was performed per sample using 2× Power SYBR Green Pre-Mix (Applied Biosystems), qPCR primer sequences available upon request. All primer pairs were verified to have sufficient amplification efficiency (>90%) and 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 using a standard program. All sample analysis was performed using the SDS 2.4 (Applied Biosystems) program. Results were normalized to the expression the reference gene, 18S and then expressed as a fold change relative to offspring born to CD M × CD F using the ΔΔCt method (i.e., fold change = 2ΔΔCt). The sexes of placental samples were determined by the presence/absence of two Y chromosome-specific transcripts (Euf235y, Uty).

Statistical Analysis

All data were expressed as means ± SE and were checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene’s test where appropriate. Statistical analysis was performed in SPSS (SPSS v. 18; SPSS, Chicago, IL) with a minimum significance level of P < 0.05. Group effects on weight gain and body composition for both sexes were analyzed by a one-way ANOVA. Fertilization rates and proportional embryo development were assessed with a Fisher’s exact test. Nanog and Oct4 stainings of blastocysts were not normally distributed and were analyzed by a nonparametric Kruskal-Wallis test. All other data were analyzed by a linear mix effects model with an LSD post hoc test. Replication, uterine horns, implantation sites, fetal sizes, and placental weights were added as covariates and mother, father, and sex as a fixed factor where appropriate.

RESULTS

HFD Increases Adiposity Without Altering Glucose Tolerance in Both Males And Females

Paternal HFD. Male mice fed a HFD for 9 wk had increased body weight (CD 29.3 ± 0.5 g vs. HFD 34.2 ± 1.0 g, P < 0.01), total sum of adipose depot weights in both absolute terms (CD 1.7 ± 0.1 vs. HFD 2.3 ± 0.2 g, P = 0.01) and relative to body weight (CD 5.2 ± 0.4 vs. HFD 6.9 ± 0.3%, P < 0.01), and increased liver weights in absolute terms (CD 1.3 ± 0.1 vs. HFD 1.8 ± 0.2 g, P < 0.05) but not relative to body weight. All other tissues measured (pancreas, kidneys, and testes) were not altered by feeding of a HFD. Additionally, HFD feeding did not alter fasting glucose levels (CD 12.0 ± 0.1 vs. HFD 12.1 ± 0.3 mmol/l, P > 0.05) or glucose tolerance in males (CD 1.039 ± 57.8 vs. HFD 1.105.7 ± 57.2 AUC, P > 0.05).

Maternal HFD. Female mice fed a HFD for 6 wk had increased body weight (CD 19.9 ± 0.3 vs. HFD 21.2 ± 0.3 g, P < 0.05) and total sum of adipose depot weights in both absolute terms (CD 0.67 ± 0.08 vs. HFD 1.27 ± 0.12 g, P < 0.01) or glucose tolerance in females (CD 1.039 ± 57.8 vs. HFD 1.105.7 ± 57.2 AUC, P > 0.05).

Maternal and Paternal HFD Reduce Fetal Size

Paternal and maternal HFD had no effect on time to mate, total implantations, or number of fetuses compared with control (P > 0.05, Table 1). Fetuses relative to total implantations were reduced (higher resorptions) only in the combined paternal and maternal feeding compared with paternal HFD (P < 0.05; Table 1). Fetal weights and lengths were reduced in all combinations of paternal and maternal HFD compared with controls (P < 0.05; Table 1), with maternal HFD irrespective of paternal HFD further reducing fetal weights compared with paternal HFD (P < 0.05; Table 1). Placental weights were decreased in combined paternal and maternal HFD compared with controls and paternal HFD (P < 0.05; Table 1). These alterations to fetal and placental weights resulted in changes to the fetal/placental weight ratio, with all diet groups displaying...
reduced ratios compared with controls \( (P < 0.05; \text{Table 1}) \), with maternal HFD reducing this ratio the most compared with paternal HFD and combined maternal and paternal HFD \( (P < 0.05; \text{Table 1}) \). Interestingly, combined maternal and paternal obesity also resulted in a number of fetuses with observed malformations, including loss of eyes and abnormal ears (Fig. 1), with no other group displaying these malformations.

### Maternal and Paternal HFD Reduces In Vivo Blastocyst Development

To determine whether the observed changes in fetal development were due to modifications in early embryo development, we flushed embryos at different time points to enable developmental assessments. There was no effect of diet on the percentage of two-cell embryos flushed from the oviduct 44 h post-hCG injection. However, maternal HFD reduced the percentage of three-cell-or-greater embryos compared with controls, suggestive of delayed development \( (P < 0.05; \text{Table 2}) \).

By 90 h post-hCG, all diet groups showed varying degrees of embryo delay compared with controls \( (P < 0.05; \text{Table 2}) \), with maternal HFD alone having a reduced percentage of expanded blastocysts, with the reciprocal increase in proportion of delayed embryos at the morula stage compared with controls \( (P < 0.05; \text{Table 2}) \). Significantly, combined paternal and maternal HFD further reduced blastocyst development with decreased percentages of expanded blastocysts with an increased percentage of arrested and delayed embryos that were less than the eight-cell stage compared with controls \( (P < 0.05; \text{Table 2}) \).

We also assessed blastocyst cell numbers, as they positively correlated with implantation, pregnancy \( (44, 59) \), and fetal health \( (47) \) and therefore might be early indicators for perturbed fetal development in response to parental HFD. Combined paternal and maternal HFD feeding reduced trophectoderm cell numbers and ICM cell numbers compared with controls and paternal HFD alone \( (P < 0.05; \text{Table 2}) \) but not as

Table 1. Effect of paternal and maternal HFD on implantation and fetal development

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD M × CD F</th>
<th>HFD M × CD F</th>
<th>CD M × HFD F</th>
<th>HFD M × HFD F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to mate (days)</td>
<td>1.7 ± 0.4</td>
<td>2.2 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Total implantations</td>
<td>9.0 ± 0.6</td>
<td>9.5 ± 0.7</td>
<td>9.0 ± 0.4</td>
<td>9.3 ± 0.6</td>
</tr>
<tr>
<td>Litter size</td>
<td>8.0 ± 0.4</td>
<td>9.2 ± 0.8</td>
<td>8.5 ± 0.6</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>Fetus per implantation (%)</td>
<td>89.4 ± 4.1ab</td>
<td>96.0 ± 2.5a</td>
<td>94.0 ± 3.4ab</td>
<td>84.6 ± 5.3b</td>
</tr>
<tr>
<td>Fetal weight (mg)</td>
<td>976 ± 14a</td>
<td>782 ± 11b</td>
<td>707 ± 14c</td>
<td>737 ± 11c</td>
</tr>
<tr>
<td>Fetal length (mm)</td>
<td>19.6 ± 0.2a</td>
<td>17.2 ± 0.2b</td>
<td>16.9 ± 0.3b</td>
<td>17.4 ± 0.2b</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>92.0 ± 2.0a</td>
<td>92.0 ± 1.8a</td>
<td>91.0 ± 2.1ab</td>
<td>87.0 ± 1.5b</td>
</tr>
<tr>
<td>Fetal/placental ratio</td>
<td>10.84 ± 0.19a</td>
<td>8.5 ± 0.15b</td>
<td>7.9 ± 0.19c</td>
<td>8.62 ± 1.50b</td>
</tr>
</tbody>
</table>

Fetal and placental data are expressed as means ± SE. Implantation and litter size data represent 6–8 females per treatment group. CD, control diet; HFD, high-fat diet; M, male; F, female. Fetal and placental data represent >30 fetuses/placentas for CD M × CD F and CD M × HFD F and >50 fetuses/placentas for HFD M × CD F and HFD M × HFD F. Different letters denote significantly distinct groups \( (P < 0.05) \).
Table 2. Effect of paternal and maternal HFD on in vivo embryo development

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD M × CD F</th>
<th>HFD M × CD F</th>
<th>CD M × HFD F</th>
<th>HFD M × HFD F</th>
</tr>
</thead>
<tbody>
<tr>
<td>44 h post-HCG</td>
<td>24.8</td>
<td>40.2</td>
<td>29.7</td>
<td>25.1</td>
</tr>
<tr>
<td>Fragmented (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two cells (%)</td>
<td>55.8</td>
<td>55.2</td>
<td>70.3</td>
<td>68.5</td>
</tr>
<tr>
<td>&gt;3 cells (%)</td>
<td>19.4ab</td>
<td>5.6ab</td>
<td>0.0</td>
<td>6.4ab</td>
</tr>
<tr>
<td>90 h post-HCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;Compacting (%)</td>
<td>33.2a</td>
<td>43.7a</td>
<td>51.6a</td>
<td>80.7a</td>
</tr>
<tr>
<td>Compacting (%)</td>
<td>20.9a</td>
<td>9.1a</td>
<td>41.4a</td>
<td>2.1a</td>
</tr>
<tr>
<td>Early blastocyst (%)</td>
<td>6.6a</td>
<td>47.2a</td>
<td>5.3a</td>
<td>5.2a</td>
</tr>
<tr>
<td>Expanded blastocyst (%)</td>
<td>39.1ab</td>
<td>1.7ab</td>
<td>11.9a</td>
<td></td>
</tr>
<tr>
<td>TCN</td>
<td>34.5 ± 1.9a</td>
<td>33.5 ± 1.7a</td>
<td>30.6 ± 2.2ab</td>
<td>27.8 ± 1.2b</td>
</tr>
<tr>
<td>TE cell number</td>
<td>25.1 ± 1.8a</td>
<td>25.1 ± 1.4a</td>
<td>23.9 ± 2.7ab</td>
<td>19.9 ± 1.4a</td>
</tr>
<tr>
<td>ICM</td>
<td>10.2 ± 0.8a</td>
<td>10.4 ± 0.7a</td>
<td>7.1 ± 0.8b</td>
<td>8.1 ± 0.5a</td>
</tr>
<tr>
<td>TE (%TCN)</td>
<td>70.6 ± 3.3</td>
<td>70.7 ± 1.5</td>
<td>75.8 ± 3.8</td>
<td>69.2 ± 2.4</td>
</tr>
<tr>
<td>ICM (%TCN)</td>
<td>30.9 ± 3.1</td>
<td>29.3 ± 1.5</td>
<td>24.2 ± 3.8</td>
<td>29.9 ± 2.4</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. TCN, total cell number; TE, trophectoderm; ICM, inner cell mass. Different letters denote significantly distinct groups at P < 0.05; n > 100 embryos at 44 h post-HCG and >50 embryos at 90 h post-HCG from 12 matings per treatment group. Cell numbers represent 20 blastocysts at 90 h post-HCG.

A percentage of total cells due to the reduction in the total cell number of blastocysts (Table 2). Maternal HFD feeding alone had no effect on blastocyst total cell numbers or trophectoderm cell numbers, but there was a reduction in ICM cell numbers compared with both controls and paternal HFD alone (P < 0.05; Table 2).

Combined Paternal and Maternal HFD Reduce Fetal Size after Embryo Transfer

Since maternal obesity has been associated with changes to the oviductal and uterine environment (71), we investigated whether the observed developmental phenotypes were due to the in utero exposure of the developing embryo to a HFD environment, changes to the gametes themselves, or a combination of both. To remove the influence of a HFD tract, embryos were collected at the zygote stage and cultured in vitro conditions to the blastocyst stage and transferred into pseudopregnant control diet-fed females. We repeated analysis of the blastocyst and fetal development as above. Blastocyst implantation rates were lower only when embryos were produced by paternal HFD alone compared with all other groups (P < 0.05; Table 3), whereas fetal development per embryo transfer was reduced only compared with maternal HFD alone (P < 0.05; Table 3). Interestingly, paternal HFD with or without maternal HFD reduced placental weights (P < 0.05; Table 4), whereas maternal HFD alone increased placental weights (P < 0.05; Table 3). This resulted in paternal HFD associated with a larger fetal-to-placental weight ratio (P < 0.05; Table 3), with maternal HFD associated with a smaller fetal/placental weight ratio (P < 0.05; Table 3). No fetal malformations were observed following embryo transfer for any group.

Paternal HFD Reduces In Vitro Embryo Development

To determine the extent to which the alterations in embryo development seen above still occurred when exposure of the embryos to the HFD was restricted to gametogenesis, embryos were removed from the tract soon after fertilization and developed in vitro. After 26 h of culture, the proportion of embryos that had cleaved, a marker of fertilization, was reduced only in paternal HFD, irrespective of maternal diet, with a reduced development seen above still occurred when exposure of the eight-cell/compacting embryos seen in combined maternal and paternal HFD (P < 0.05; Table 4). This delay in embryo development was still evident after 74 and 91 h of culture by paternal HFD, irrespective of maternal diet, with a reduced proportion of on-time blastocyst development compared with controls (P < 0.05; Table 4). In contrast, the delay in embryo development seen at 43 h was no longer evident in embryos

Table 3. Effect of paternal and maternal HFD on implantation and fetal development after embryo transfer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD M × CD F</th>
<th>HFD M × CD F</th>
<th>CD M × HFD F</th>
<th>HFD M × HFD F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation/ET (%)</td>
<td>87.0ab</td>
<td>68.6b</td>
<td>91.9a</td>
<td>80.8a</td>
</tr>
<tr>
<td>Fetal development/ET (%)</td>
<td>34.5ab</td>
<td>25.0a</td>
<td>46.1a</td>
<td>24.4a</td>
</tr>
<tr>
<td>Fetal development/implantation (%)</td>
<td>44.1ab</td>
<td>36.4a</td>
<td>56.1a</td>
<td>30.1a</td>
</tr>
<tr>
<td>Fetal weight (mg)</td>
<td>891 ± 26a</td>
<td>901 ± 48ab</td>
<td>947 ± 32a</td>
<td>796 ± 36a</td>
</tr>
<tr>
<td>Fetal length (mm)</td>
<td>19.9 ± 0.3a</td>
<td>20.2 ± 0.5a</td>
<td>20.1 ± 0.3a</td>
<td>18.1 ± 0.4a</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>118 ± 4.0a</td>
<td>89 ± 7.0b</td>
<td>155 ± 5.0b</td>
<td>102 ± 6.0b</td>
</tr>
<tr>
<td>Fetal/placental ratio</td>
<td>7.78 ± 0.30a</td>
<td>10.17 ± 0.30a</td>
<td>6.31 ± 0.34a</td>
<td>8.02 ± 0.40a</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Implantation data represent 12 recipient females per treatment group, with 60 embryos transferred per group. ET, embryo transfer. Fetal and placental data represent >10 fetuses/placentas per treatment group. Different letters denote significantly distinct groups (P < 0.05).
Table 4. Effect of paternal and maternal HFD on in vitro embryo development

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD M × CD F</th>
<th>HFD M × CD F</th>
<th>CD M × HFD F</th>
<th>HFD M × HFD F</th>
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<tr>
<td>26 h of culture</td>
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<tr>
<td>2 cells (%)</td>
<td>88.6a</td>
<td>67.9b</td>
<td>84.0a</td>
<td>68.7b</td>
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<td>43 h of culture</td>
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<tr>
<td>≥8 cells (%)</td>
<td>74.0a</td>
<td>59.3b</td>
<td>54.1b</td>
<td>35.1c</td>
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<td>Compaction (%)</td>
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<td>23.5b</td>
<td>28.9b</td>
<td>6.3b</td>
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<td>74 h of culture</td>
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<tr>
<td>≥Early blastocyst (%)</td>
<td>82.8a</td>
<td>69.5b</td>
<td>82.2a</td>
<td>68.4a</td>
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<tr>
<td>Total blastocysts (%)</td>
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<td>76.1b</td>
<td>88.9a</td>
<td>81.9b</td>
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<td>Expanded blastocyst (%)</td>
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<td>29.8a</td>
<td>5.9b</td>
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<td>Hatching blastocysts (%)</td>
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<td>44.4b</td>
<td>78.9a</td>
<td>47.6b</td>
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<tr>
<td>TCN</td>
<td>73.7 ± 2.1a</td>
<td>70.1 ± 2.3a</td>
<td>74.5 ± 3.1a</td>
<td>69.1 ± 1.9b</td>
</tr>
<tr>
<td>TE cell number</td>
<td>60.5 ± 1.8</td>
<td>56.6 ± 2.0</td>
<td>62.8 ± 2.8</td>
<td>59.3 ± 1.7</td>
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<td>ICM</td>
<td>13.0 ± 0.5a</td>
<td>13.0 ± 0.6a</td>
<td>11.4 ± 0.8b</td>
<td>9.7 ± 0.4b</td>
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<td>TE/TCN (%)</td>
<td>81.7 ± 0.7a</td>
<td>79.6 ± 0.8a</td>
<td>84.2 ± 0.9a</td>
<td>85.7 ± 0.6b</td>
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<tr>
<td>ICM/TCN (%)</td>
<td>17.7 ± 0.6a</td>
<td>18.7 ± 0.7a</td>
<td>15.2 ± 0.9b</td>
<td>13.9 ± 0.6b</td>
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<td>115 h of culture</td>
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<tr>
<td>Epiblast cell number (Nanog/Oct4 positive)</td>
<td>3.2 ± 0.2a</td>
<td>1.2 ± 0.4a</td>
<td>3.3 ± 0.3a</td>
<td>1.2 ± 0.3b</td>
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<tr>
<td>Epiblast cells: ICM (%)</td>
<td>29.2 ± 2.1a</td>
<td>14.3 ± 3.6a</td>
<td>29.1 ± 3.1a</td>
<td>14.4 ± 3.2b</td>
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<tr>
<td>Blastocysts with no epiblast cells (%)</td>
<td>6.0a</td>
<td>33.0b</td>
<td>7.0a</td>
<td>49.0b</td>
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Data are as means ± SE and represent proportion of fertilized embryos that reached each developmental milestone; 26-h development data are a proportion of the number of 1-cells collected. From 43 h of development, all proportion data are relative to the numbers of 2-cell embryos at 26 h. Blastocyst cell numbers were determined after 91 h of embryo culture; epiblast cell numbers were determined after 115 h of embryo culture. Different letters denote significantly distinct groups at P < 0.05; >200 embryos per treatment group for developmental measures; >25 embryos per treatment for cell number measures.

Produced by maternal HFD feeding after 74 and 91 h of embryo culture (Table 4).

Total cell numbers and ICM numbers were reduced only in combined paternal and maternal HFD (P < 0.05; Table 4), whereas the proportion of ICM to total cell number was reduced in blastocysts from maternal HFD (P < 0.05; Table 4). Although there were no differences in TE cell numbers across treatment groups (Table 4), as a proportion of total cell number, TE cells were increased by maternal HFD with any combination of paternal diet (P < 0.05; Table 4). Epiblast cells both, as the number and as a percentage of total ICM, were reduced in blastocysts produced by paternal HFD compared with controls (P < 0.05; Table 4), and this was not further altered by mating with a HFD mother (P < 0.05; Table 4). In addition, the proportion of blastocysts lacking epiblast cells were increased by paternal HFD, although no further change were seen with two HFD parents (P < 0.05; Table 4).

Cumulatively these results show that fetal and blastocyst development are still perturbed by paternal HFD even when removed from the reproductive tract, suggesting that preconception changes to oocytes and sperm are a substantive component influencing the fetal phenotypes.

Maternal and Paternal HFD Differentially Alter Mitochondria in Two-Cell Embryos

We (87, 88) have previously shown that mitochondrial markers (i.e., MitoTracker and JC-1) are altered in oocytes of obese female mice; thus, we examined whether these changes persist in the developing embryo. Maternal HFD alone increased abundance of Mitotracker staining compared with controls (P < 0.01; Fig. 2), whereas paternal HFD alone decreased abundance of Mitotracker staining compared with controls (P < 0.01; Fig. 2). Interestingly, combined maternal and paternal HFD increased Mitotracker abundance compared with controls and paternal HFD alone (P < 0.01; Fig. 2); however, it decreased Mitotracker abundance compared with maternal HFD alone (P < 0.01; Fig. 2).

Paternal HFD Increases Active Chromatin Marks in Two-Cell Embryos

Paternal diet-induced obesity has been shown to alter epigenetic chromatin marks (H3K27me3) in Drosophila sperm, which persist in 12-h embryos and into adult offspring tissues (68). We thus wanted to determine whether a paternal HFD in our mouse model also disrupted active and repressive chromatin marks in the early embryo. Paternal HFD alone increased the ratio of active (H3K4me3) to repressive (H3K27me3) chromatin marks in two-cell embryos compared with controls (P < 0.05; Fig. 3) and maternal HFD alone (P = 0.08; Fig. 3). Maternal HFD alone had no effect on chromatin marks in two-cell embryos compared with controls (Fig. 3), it was not statistically significant compared with any group (P > 0.05; Fig. 3).

Maternal HFD Impairs mtDNA Copy Number in Fetal Livers

mtDNA copy number has been previously shown to be reduced in oocytes of obese female mice and to persist into the blastocysts and fetal tissue (88). To investigate whether this occurred in response to paternal or combined diet-induced obesity we examined fetal livers from all diet crosses. Maternal HFD, irrespective of paternal diet, led to decreased mtDNA copy number in fetal livers compared with controls and paternal HFD alone (P = 0.08; Fig. 4A), whereas paternal HFD did not alter mtDNA copy number in...
fetal livers compared with controls ($P > 0.05$; Fig. 4A). There was no effect of fetal sex on mtDNA copy number ($P > 0.05$; data not shown).

**Paternal and Maternal HFD Alter Placental Gene Expression**

Either maternal or paternal HFD in isolation has been shown to change the transcriptome of the developing placenta (5, 8, 46, 75). Thus, we determined whether there were additive effects of combined paternal and maternal HFD on placental gene expression during gestation. We focused on imprinting, metabolic, and mitochondrial genes that are associated with transgenerational programming of metabolic health. *Glut1* gene expression was increased in placentas from male offspring from all maternal and paternal HFD diet combinations compared with controls ($P < 0.05$; Fig. 4B), whereas gene expression in placentas from females were less dramatically affected, although maternal HFD generally decreased gene expression ($P < 0.05$; Fig. 4B). *Glut3* gene expression was increased in placentas of males of combined maternal and paternal HFD compared with controls ($P < 0.05$; Fig. 4C); however, expression was not different from either maternal or paternal HFD alone ($P > 0.05$; Fig. 4C). There was no effect of gene expression on imprinting genes *H19, Igf2*, and *Igf1r* in placentas from male offspring for any group; however, maternal HFD, irrespective of paternal HFD, decreased gene expression of these imprinting genes compared with controls and paternal HFD alone in placentas from females ($P < 0.05$; Fig. 4, D–F). Amino acid transporter *Slc38a2* gene expression was increased by paternal HFD in placentas of both males and females compared with controls ($P < 0.05$; Fig. 4G), while maternal HFD also increased expression in placentas from female offspring compared with controls ($P < 0.05$; Fig. 4G). Mitochondrial gene *Nrf1* expression was decreased in placentas from females but not males by paternal HFD alone compared with both controls and maternal HFD alone ($P < 0.05$; Fig. 4H), and although combined paternal and maternal HFD showed decreased expression, this was not different from any group ($P > 0.05$; Fig. 4H). Gene expression of *Slc38a4, Nrf2, Terf1a, Tfam*, or *Vegf* in placentas was unaltered as a result of maternal and/or paternal HFD feeding for both sexes ($P > 0.05$; data not shown).

**DISCUSSION**

Combined parental obesity is becoming increasingly common in reproductive-age adults in Western civilizations. However, the impact of having two overweight/obese parents on pregnancy and fetal development has received limited attention. While a rodent study demonstrated the amplification of metabolic disease in offspring from combined paternal and maternal obesity (67), whether this was as a result of changes to early embryo development and function is unknown. Here, we show that parental obesity reduces fetal and placental weights without altering pregnancy establishment and is not dependent on an in utero exposure to a HFD. Furthermore, these changes to offspring phenotypes may result from perturbed early embryo and fetal health that manifest as reduced embryo developmental competency, reduced blastocyst cell numbers, impaired mitochondrial function, and alterations to active and repressive embryonic chromatin marks, resulting in aberrant placental gene expression and reduced fetal liver mtDNA copy numbers.

**Combined Paternal and Maternal Obesity Alters Fetal and Placental Development**

Combined paternal and maternal obesity further reduced fetal and placental weights compared with maternal or paternal obesity alone following both natural mating and embryo transfer, suggesting that the changes were not dependent on in utero HFD exposure. Being small for gestational age is associated with increased risk of noncommunicable diseases in offspring (4, 34), with combined paternal and maternal obesity previously shown to amplify metabolic syndrome and liver damage in a rodent model (67). Both maternal and paternal obesity have independently been shown to perturb gamete quality; therefore, it is likely that fetal and placental phenotypes were due to the compounded effects of combined parental obesity. For example, female obesity has been shown to alter the ovarian environment, with changes to intrafollicular insulin and triglycerides (74, 83, 84), increased expres-
sion of lipoprotein receptors (74), and subsequent lipid accumulation, endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis in cumulus oocyte complexes (87, 88). Male obesity has been shown to change the testicular and epididymal microenvironments, altering expression of oxidative stress and inflammatory genes and proteins (10, 50), and subsequently causing mitochondrial dysfunction, oxidative stress, DNA damage, and chromatin changes in mature sperm (3, 38, 42, 68, 69, 81).

In addition to reduced fetal and placental sizes, it was observed that fetuses from combined paternal and maternal obesity also displayed increases in congenital malformation, including loss of eyes and abnormal ear attachment, compared with any other group (Fig. 1). In humans, pre-conception maternal obesity is associated with increased odd ratios (OR) for birth defects, including neural tube defects (OR 1.87), cardiovascular abnormalities (OR 1.30), orofacial clefts (OR 1.23), and anorectal atresia (OR 1.48) (79). However, in many of the studies the male partner BMI was not recorded; therefore, whether these increases in birth defects were as a result of maternal obesity alone or in conjunction with paternal obesity or another factor remains unknown.

While we saw similar effects on fetal growth in both our HFD in utero exposure and our in vitro models from combined paternal and maternal obesity, we did see a worsened fetal and early embryo phenotype from exposure to a HFD in oviduct and uterine tract. As maternal obesity is associated with changes to the uterine environment including increased inflammatory markers (71), and changes to uterine fluid composition (11, 91), it is likely that this perturbed environment is acting as a “second hit” further perturbing fetal growth. Interestingly, we also saw a worsened early embryo phenotype from in vitro culture of embryos produced from paternal HFD, indicating that the in vitro culture itself and/or the superovulation protocol was interacting with the paternal HFD. This is not surprising, as embryos produced from in vitro culture and or superovulation have an altered health compared with in vivo derived embryos (36), and again potentially applying a second stress that unmasks the paternal contribution.

Fig. 3. Effect of paternal and maternal HFD feeding on active and repressive chromatin marks in 2-cell embryos. Epifluorescent representative pictures of 2-cell embryos stained with H3K4me3 (red), H3K27me3 (green), Hoechst (blue), and phase contrast (bright field) (A) and ratio of active to repressive chromatin marks in 2-cell embryos relative to 2-cell embryos generated from CD males and females (B). Data represent 20 embryos per group. Different letters denote significance at $P < 0.05$; *different from HFD M × CD F at $P = 0.08$. 
Fig. 4. Effect of paternal and maternal HFD feeding on mtDNA copy number in fetal livers and placental gene expression. A: mtDNA copy number in combined male and female fetal livers and relative gene expression of both male and female placentas expressed as ΔΔCt vs. placentas from control fathers and mothers. B–H: Glut1 (B), Glut3 (C), H19 (D), Igf2 (E), Igf2r (F), Slc38a2 (G), and mNrf1 (H). Data represent 10 fetus/placentas from 5 matings for CD M × CD F and CD M × CD F; 12 fetus/placentas from 6 matings for HFD M × CD F and HFD M × HFD F. Different letters denote significance at P < 0.05; *different from HFD M × HFD F at P = 0.08.
Maternal obesity is associated with reduced mtDNA copy number in oocytes, blastocysts, and fetal tissues (88). Post-fertilization, oocyte-derived mitochondria are segregated into each embryonic daughter cell until the blastocyst stage, at which point the pluripotent ICM cells establish a threshold mtDNA set point from which they commence mtDNA replication in a differentiation-specified manner (37, 78). Because oocyte-derived mitochondria give rise to the entire complement of mitochondria in offspring tissues, their transmission, replication, and inheritance are tightly regulated; however, this process is sensitive to maternal metabolic status and stress such as obesity (37, 78). Similar to what we found in a genetic mouse model of female obesity (88), we found that mtDNA copy number was reduced in fetal livers of female and male offspring from maternal HFD exposure, with levels not being further reduced by the addition of paternal obesity. In a number of tissues mtDNA copy is tightly associated with different types of cancer (49, 57, 58), cellular telomere length (82), and tissue-specific processes (15); therefore, disruption of mtDNA copy number is linked with a number of diseases and mortality (2, 65). As offspring from female rodents fed high-calorie diets (before conception through to lactation) are smaller and have metabolic syndrome, similar to phenotypes reported in offspring generated in our study and others (35, 52), reductions in mtDNA content in fetal tissues perhaps could be one of the mechanisms for a later predisposition to chronic disease. Interestingly, mtDNA copy number is also reduced in human placentas of mothers who are obese (31).

Because we see an accumulation of both maternal and paternal embryonic and fetal phenotypes in our combined obesity group, we know that there must be paternally driven changes in the early embryo contributing to the overall effect. Because father-to-embryo transmission excludes gestational effects, mechanistically, imprinting, altered DNA methylation, histone modifications, and noncoding RNA changes in sperm or alterations to seminal plasma composition, which can influence the signaling of the female reproductive tract, have been implicated in paternal nongenetic transmission (9, 53). In Drosophila, consumption of a high-sugar diet by fathers alters the chromatin state of metabolic dependent genes in offspring, which persisted into embryos and which were originally altered in sperm (68). We therefore assessed the global chromatin status, focusing on active (H3K4me3) and repressive (H3K27me3) chromatin marks in two-cell embryos. Interestingly, the ratio of active to repressive chromatin marks was altered in two-cells from obese fathers, which was not amplified by maternal obesity. Trimethylation of H3K4me3 and H3K27me3 plays important roles in embryonic genome activation and blastocyst cell lineage segregation, with decreased global levels of H3K4me3 present both prior to and after the time of major genomic activation in embryo (24); this is consistent with embryos produced by paternal obesity having reduced first and second cleavage events and reduced numbers of epiblast cells (6, 7, 54). However, given the limitations of a global antibody approach, further studies are required to confirm potential changes in gene expression.

It has been previously demonstrated that the placenta can adapt to environmental perturbations by altering its size to regulate nutrient supply to the fetus (61). As both in vivo- and vitro-derived fetuses and placentas were reduced in size with combined paternal and maternal obesity, and since both pater-
nal and maternity obesity alone alter placental gene expression (5, 8, 46, 75), we established whether key imprinting, growth, nutrition, and mitochondrial specific gene regulation were further impaired when both parents were obese. We observed sex-specific effects on placental gene expression that were expected given the numerous reports of sex-specific phenotypes in offspring in models of maternal and paternal obesity (18, 19, 22, 23, 63, 66, 67). Glucose is transferred from the maternal blood to the fetus via the placenta through glucose transporters (43). However, in growth-restricted fetuses similar to what we see in our maternal and paternal obesity in vivo models, both GLUT1 and GLUT3 gene expressions are known to be upregulated in human placentalas (33). This has been proposed to occur as an adaptive mechanism to increase nutrient transport in late gestation to try and increase fetal growth (33). In addition, increases in gene expression of the amino acid transporter Slc38a2 is associated with faster-growing fetuses (14). Paternal HFD increased Slc38a2 expression in placentas from males, and placentas from females had increased expression from all diet combinations compared with controls. As both maternal obesity and paternal obesity are associated with increased offspring size at birth (13, 23, 55, 72), this may suggest that an increased nutrient flow through the placenta in very late gestation that increases the growth rate of the initially smaller fetuses.

**Combined Effect on Fetal Growth from Maternal and Paternal Obesity Hypothesis**

Although we did not observe an amplified effect on early embryonic health as a result of combined paternal and maternal obesity, we did see an accumulation of both paternal and maternal negative influences. Maternal obesity alters both the nuclear and cytoplasmic content of the oocyte, resulting in mitochondrial dysfunction and increased lipid accumulation, which were inherited by the early embryo and fetus. In contrast, paternal obesity has been shown to alter the chromatin status of sperm, altering the methylation status of both DNA and chromatin, which also persists into the developing embryo. During fertilization events, the early embryo undergoes substantial remodeling of the paternal and maternal derived genetic and epigenetic information (73) to establish a totipotent embryo; therefore, any changes or alterations, as a result of an environmental insult, during these key developmental time frames could permanently alter phenotypes in offspring. As replication and pronuclear repair of both the paternal and maternal genomes after fertilization relies solely on maternal derived machinery and mitochondrial derived substrates (27), alterations to sperm chromatin state from paternal obesity coupled with impaired mitochondrial quality from maternal obesity may result in a further delay in genome activation. This could trigger downstream consequences of further impaired cell allocations and embryo development, transcriptional changes in the blastocyst and subsequent placenta, which in turn affect fetal growth, altering the growth trajectory of offspring and increasing susceptibility to adult chronic disease (45).

Our findings demonstrate that the additive effects seen in offspring phenotypes as a result of combined paternal and maternal obesity (16, 67) are likely due to the accumulation of both paternal and maternal effects on embryo and fetal development. Although we have begun to elucidate the underlying mechanisms behind this programming effect, further understanding of both the maternal and paternal genetic and cytoplasmic interactions during this early developmental time frame will be vital for understanding how developmental programming is regulated and for the proposition of interventions to mitigate their effects.

**ACKNOWLEDGMENTS**

We thank Vitrolife for the kind donation of media for embryo culture and rapid-I for vitrification, and Lauren Sandeman for technical assistance.

**GRANTS**

This research is supported by a National Health and Medical Research Council (NHMRC) grant awarded to M. Lane. M. Lane is a recipient of an NHMRC Senior Research Fellowship. N. McPherson and T. Fullston are recipients of an NHMRC Early Career Fellowship. R. Robker is the recipient of an NHMRC Career Development Fellowship. V. Bell acknowledges the support of the Freemasons Lodge St Albans Research Scholarship.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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