Preconception diet or exercise intervention in obese fathers normalizes sperm microRNA profile and metabolic syndrome in female offspring

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This is an important question, because in western societies, the incidence of obesity has more than doubled in children and tripled in adolescence (44). Obesity increases the risk of type 2 diabetes mellitus (T2DM), cancer, stroke, heart disease, and osteoarthritis in adulthood (49). It is widely accepted that increased maternal BMI and hyperglycemia during gestation or lactation is associated with obesity and T2DM in children through developmental programming separate from shared genetics or postnatal environment (27, 48). Recent studies now suggest that paternal metabolic health at conception can also impact children’s health, with obese fathers more likely to father an obese child (35). In rodents, diet-induced male obesity with or without diabetes induces a worsened metabolic phenotype in their female offspring, with glucose intolerance in female offspring due to pancreatic islet dysfunction and white adipose tissue dysfunction or insulin resistance and obesity, with some consequences evident across two generations (18, 45, 46). However, whether short-term lifestyle interventions in the obese father could rescue these programmed metabolic phenotypes in female offspring is currently unknown.

This is an important question, because in western societies, greater than 70% of reproductive-aged men are overweight or obese (e.g., 74% in the US) (15, 44). Weight loss via diet and exercise interventions in obese men improves glucose control and insulin action (9), alters epigenetic marks (i.e., DNA methylation and microRNA) in their leukocytes (41, 50), and also improves their reproductive function (i.e., sexual function, hormone profiles, and basic sperm parameters) (23). Previously, we have shown that diet and/or exercise interventions in founder male mice, which are obese due to a 10-wk high-fat diet, reduce their excess adiposity and improve their metabolic profile and sperm function and reverse the adverse outcomes for early embryonic/fetal development (37, 38, 53). Because of the worsened metabolic phenotype of female offspring from the original two rodent studies, we investigated whether these same interventions in obese founder males could improve the metabolic health of their female offspring. Here, we show that short-term diet and exercise intervention in diet-induced obese founder male mice improves their metabolic health and/or adiposity and prevents or ameliorates programmed insulin resistance and large adipocytes in their female offspring, concomitant with a degree of normalization of sperm microRNA content.

MATERIALS AND METHODS

Founder Animals and Diet

Five-week-old male C57BL6 mice (n = 40) were randomly assigned to one of two diets for an initial feeding period of 9 wk (pre intervention): 1) control diet (CD) (SF04-057; Specialty Feeds, Perth, Australia) or 2) a high-fat diet (HFD) containing 21% fat and nutrient matched (SF00-219; Specialty Feeds) (Table 1). The HFD used in the study has previously been shown to increase adiposity (7, 10, 42, 54). After the initial feeding period, males fed the HFD were then allocated to one of the following interventions for an additional period of 9 wk: 1) continuation of a HFD (HH) (n = 8), 2) change to a CD (HC) (n = 8), 3) continuation of a HFD with exercise (HE) (n = 8), and 4) change to a CD with exercise (HCE) (n = 8). Mice allocated to the CD during the initial feeding period were also fed a CD during the intervention period to be used as a baseline control (CC) (n = 7). The length of exposure to these interventions has previously been shown to reduce adiposity in those males that undergo diet interventions and to improve metabolic parameters in those males that undergo exercise or exercise combined with diet interventions (53). Animals were housed individually in a 12:12-h light-dark cycle for the entire study and fed ad libitum. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

Exercise Intervention (Swimming)

The swimming exercise regime was conducted as described previously (53). Briefly, male mice were placed into tanks containing warm water at a constant temperature of 32 ± 1°C to swim freely for the set time period. For the first 2 wk, mice swam for 3 × 15-min periods over 7 days. This allowed time for the mice to become accustomed to...
the exercise regime and the swimming tank. For the remainder of the intervention (6 wk), mice swam for 3 x 30-min training sessions each week to simulate light exercise. Swimming sessions were supervised, and the number of mice per swim session ($n = 4$) was kept low to avoid “gang swimming.”

**Founder Body Composition**

Founder body weights were recorded weekly during both the initial and postintervention periods. At *preintervention week 9* and *intervention week 8*, whole body compositions of adiposity were measured by a dual-emission X-ray absorptiometry machine (DEXA; Piximus; Ge Lunar), as described previously (43). At 23 wk of age, adiposity (gonadal adiposity, omental adiposity, retroperitoneal adiposity, peritoneal adiposity, and dorsal adiposity), testes, seminal vesicles, liver, kidneys, and pancreas were collected and weighed by the same individual who was blinded to treatment group.

**Founder Glucose Tolerance Test and Insulin Tolerance Test**

At *intervention week 7*, a glucose tolerance test (GTT) was performed after 6 h of fasting by intraperitoneal (ip) injection of 2 g/kg of a 25% d-glucose solution. Insulin tolerance test (ITT) was performed at *intervention week 8* during a fed state by ip injection of 0.75 IU of human insulin (Actaprid; Novo Nordisk, Bagsvaerd, Denmark). Tail blood glucose concentrations were measured using a glucometer (Hemocue, Angelholm, Sweden) at time points 0 (prebolus basal), 15, 30, 60, and 120 min. Data were expressed as mean blood glucose concentration per group as area under the curve (AUC) for glucose and area above the curve (AAC) for insulin.

**Natural Mating to Produce F1 Females**

At *intervention week 7* (21 wk of age), founder males had the opportunity to mate with two randomly chosen cycling normal-weight 10-wk-old C57BL6 females during a maximum period of 8 days. Female mice were exposed to founder males during the dark cycle only and were separated from the males and maintained on standard chow during the light cycle. Successful mating was assessed the following morning by the presence of a vaginal plug. After successful mating, female mice were group housed until day 15 of pregnancy, where they were housed individually until the offspring were weaned.

Mothers were maintained on standard chow during pregnancy and postbirth. Mothers were allowed to pup, and at weaning (day 21 of life) female offspring were separated from their mothers and group housed independently of founder treatment and maintained on standard chow. For each independent measurement of metabolic health, one female was sampled per litter, as described previously (33), to reduce litter effects.

**F1 Female Offspring Health**

Preweaning body weights were recorded on days 5, 7, 10, 12, 14, and 21 postbirth and individual pups tracked. Postweaning individual female body weights were recorded up until 18 wk of age. At 8 and 16 wk of age, 10 females from 10 litters representative of seven founders per treatment group underwent a DEXA, as described above. At 10 and 18 wk of age, an additional seven to eight females from seven to eight litters representative of at least five founders per treatment group underwent a full postmortem where adipose depots (gonadal, omental, retroperitoneal, peritoneal, and dorsal), liver, kidneys, and pancreas were collected, weighed, and performed blinded by the same individual.

**F1 Female GTT and ITT**

At 8–9 and 16–17 wk of age, 10 females from 10 litters representative of seven founders per treatment group underwent repeated GTT and ITT assessments, as described above.

**F1 Female Insulin Response During a GTT**

At 16 wk of age, seven females from seven litters representative of four founder males underwent insulin testing during a GTT. At time points 0 (prebolus basal), 15, 30, and 60 min an additional 50 μl of blood was obtained via the tail vein using a paster pipette. Whole blood was centrifuged at 4,000 rpm for 5 min, and between 5 and 10 μl of plasma was removed and frozen at −20°C until further testing. Insulin concentrations were determined by an Ultra-Sensitive Mouse Insulin ELISA Kit (no. 90080; Crystal Chem, Downer Grove, IL) as per the manufacturer’s instructions. Data were expressed as mean plasma insulin concentration per group as area under the curve (AUC) and insulin secretion relative to the glucose stimulus at 0, 15, 30, and 60 min.

**Metabolites and Hormone Analysis**

At 9 wk postintervention for founder males and at 10 and 18 wk of age for F1 females, overnight fasting blood plasma was collected at postmortem by a cardiac puncture under anesthetic with 2% isoflurane (1-chloro-2,2,2-trifluoroethylidifluoromethylether; Veterinary Companies of Australia, Kings Park, Australia) for founders or Avertin (2,2,2-trimethyl-1-propanol; Sigma-Aldrich, St. Louis, MO) at 5% body weight for F1 females. Plasma cholesterol, free fatty acids (FFA), glucose, and triglyceride concentrations were measured on a Cobas Integra 400 Plus automated sampler system (Roche, Basel, Switzerland). Plasma insulin, leptin, adiponectin, corticosterone, and C-reactive protein (CRP) levels were measured by either an Ultra-Sensitive Mouse Insulin ELISA Kit (no. 90080; Crystal Chem), a mouse leptin ELISA kit (no. 90030; Crystal Chem), a Quantikine ELISA mouse adiponectin/Acrp30 kit (no. MRP300; R & D Systems, Minneapolis, MN), a corticosterone ELISA kit (no. KA0468; Abnova, Taipei City, Taiwan), or a CRP mouse ELISA kit (no. KA2467; Abnova) as per the manufacturers’ instructions.

**Histology of F1 Female Gonadal Adiposity**

Gonadal adiposity was chosen for histology analysis, as it was the most affected adipose depot in female offspring, as per our previous studies (16). F1 female gonadal adiposity was fixed overnight in 4% formaldehyde and stored in 70% ethanol until further use. Gonadal adiposity was embedded in wax using standard methods, and 7-μm sections were cut and heat-fixed onto Superfrost slides (Menzel-Glaser, Brauschwig, Germany). Each slide contained four 7-μm sections that were 50 μm apart. Slides were dewaxed, rehydrated in ethanol dilutions, and stained with hematoxylin and eosin as per standard methods. Slides were mounted in DPX mounting medium and imaged using a NanoZoomer slide scanner (Hamamatsu Photonics; Sunayama-cho, Naka-ku, Japan). The areas of 100 adipocytes

### Table 1. Composition of rodent diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CD (SF04-057), Low-Fat Control</th>
<th>HFD (SF00-219), Harlan-Teklad TD88137 Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose, g/100 g</td>
<td>34.1</td>
<td>34.1</td>
</tr>
<tr>
<td>Casein (acid), g/100 g</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Canola oil, g/100 g</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Clarified butter, g/100 g</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>Cellulose, g/100 g</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Wheat starch, g/100 g</td>
<td>30.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Minerals, g/100 g</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Digestible energy, MJ/kg</td>
<td>16.1</td>
<td>19.4</td>
</tr>
<tr>
<td>Digestible energy from lipids, %</td>
<td>21.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Digestible energy from protein, %</td>
<td>14.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

CD, control diet; HFD, high-fat diet.
Adiposity levels. Correlations of female offspring measurements to founder metabolic measurements were determined by a partial correlation controlling for litter size and founder ID to control for multiple observations for individual founders.

RESULTS

Adiposity and Metabolic Health is Improved in Offspring as a Response to Interventions in Founders

No intervention (HH). FOUNDERS. Consistent with our previous reports (53), feeding males a high-fat diet (HH) for 18 wk increased weight (48%), adiposity (39%), plasma cholesterol (30%), insulin (ITT, 67%), leptin (75%), corticosterone (15%), and proinflammatory CRP (21%, \( P < 0.05 \); Table 2). Diet-induced obesity in founders did not alter time to mate, gestational length, litter size, or offspring sex ratio compared with CC founders (Table 3); however, HH founders had a reduced number of successful matings compared with CC founders (−31%, \( P < 0.05 \); Table 2).

OFFSPRING. Female offspring born to HH founders were heavier on postnatal day 12 compared with female offspring from CC founders, which persisted until weaning (\( P < 0.05 \); Fig. 1), resulting in HH female offspring gaining more weight during the preweaning period compared with CC offspring (\( P < 0.05 \); Fig. 1D). Consistent with our previous study in mice (18), HH female offspring accumulated more site-specific renal adipose depots as a percentage of total body weight, with a 19% increase seen at 18 wk compared with CC female offspring (\( P < 0.05 \); Fig. 2A). HH female offspring had hypertrophic adipocytes in their gonadal adipose depot (\( P < 0.05 \); Fig. 2, D and G) and reduced plasma adiponectin at 10 wk compared with CC female offspring (\( P < 0.05 \); Fig. 2F), which persisted at 18 wk (\( P < 0.05 \); Fig. 2, D and E). HH female offspring also had increased plasma triglycerides at both 10 and 18 wk compared with CC female offspring (\( P < 0.05 \); Fig. 2F). HH female offspring had normal fasting plasma glucose and glucose tolerance at 8 and 16 wk compared with CC offspring (Tables 4 and 5 and Fig. 3, A–C). However, HH female offspring were insulin resistant at both 9 and 17 wk (\( P < 0.05 \); Fig. 3, D–F) and secreted more insulin during a glucose challenge compared with CC female offspring at 16 wk (\( P < 0.05 \); Fig. 3, G–J) despite no differences in fasted plasma insulin concentrations (Tables 4 and 5).

Diet-alone intervention (HC). FOUNDERS. Diet-alone intervention in obese founders normalized their weight, adiposity, plasma insulin, cholesterol, and leptin but did not their increased plasma corticosterone (+26%) or CRP (+15%) compared with CC founders (Table 2). HC did not alter time to mate, gestational length, or offspring sex ratio (Table 3); however, it did improve the proportion of successful matings compared with HH founders (+21%; Table 3) and increased litter size compared with both CC and HH founders (\( P < 0.05 \); Table 3).

OFFSPRING. HC female offspring had reduced body weight during the neonatal and postnatal period up until 4 wk of age compared with female offspring of CC and HH founders (\( P < 0.05 \); Fig. 1A); however, they exhibited an increase in preweaning weight gain compared with CC offspring (Fig. 1D). This smaller preweaning size was associated with increased adipose depots at 8 wk compared with both HH (gonadal and dorsal) and CC (gonadal) female offspring (\( P < 0.05 \); Fig. 2, B and C, and Table 4), which was normalized by 18 wk (Fig. 2, B and C). Adipocyte cell size was reduced compared with HH off-

MicroRNA Analysis of Founder Sperm

Sperm were obtained from the vas deferens. Sample purity was assessed using cell count, whereby samples were confirmed to be >99% pure. Total RNA was isolated from 4 to 8 × 10⁶ sperm with TRI reagent, using glycogen as a RNA carrier, and concentration and quality of RNA were assessed by spectrophotometry (Nanodrop; Thermo Scientific, Waltham, MA). As the transmission of the worse metabolic health phenotypes reported by paternal obesity (10 wk on diet) through subsequent generations described in our original study (grandfather to mother to son) (18), X-linked microRNA targets were chosen from previous microRNA expression profiling of founder CD and HFD sperm (15 wk of age, 10 wk on diet, matched to the original study) by a 384-well microfluidic TagMan Rodent MicroRNA Array card version 3.0 amplified on a 7900 HT Real Time PCR system (data not shown). Differentially expressed X-linked microRNAs were chosen based on ranking using LIMMA in R after raw CT data were quantile normalized using the normQpcrQuantile function of the R qPCRnorm package (data not shown). MicroRNAs were chosen if they displayed a \( P \) value of ≤0.05 with a fold change of ≥1.5 or up to −1.5. Validation of these microRNAs in founder sperm postintervention (18 wk on diet) was performed by qRT-PCR using multiplexed TaqMan primers (Applied Biosystems, Foster City, CA) and the RT product premultiplied with Megaplex PreAmp Primers (Applied Biosystems) on a 7900 HT Real Time PCR system, including no template control and no reverse transcription control. MicroRNA expression fold change in samples was determined by \( \Delta \Delta C_T \), with data normalized to the geometric mean of mmu-mir-10a, which was determined to be invariable and ubiquitous endogenous control by cel-miR normalization of the original array data (data not shown). All data are expressed as fold change relative to controls (CC).

Statistics

All data were expressed as means ± SE and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene’s test. Statistical analysis was performed in SPSS (SPSS version 18; SPSS, Chicago, IL), with AUC and AAC calculated in GraphPad Prism (GraphPad Software version 6; GraphPad, San Diego, CA). A \( P \) value of <0.05 was considered to be significant, and the statistical analysis was accepted if power of the model was ≥80%.

Founder metabolites, body composition, weight gain/loss, and sperm microRNAs were measured using a linear mixed-effect model. In the model, cohorts of animals were fitted as a covariate.

Founder mating rates, time to mate, gestational length, litter size, and sex ratios were expressed per father and analyzed by a Fisher’s exact test.

F1 preweaning weights were analyzed using repeated-measures ANOVA. In the model, father ID and mother ID were included as a random effect to adjust for dependence in results between offspring from the same father and mother and litter size as a fixed variable to compare litter size variations between and within treatments.

F1 adult offspring measurements were analyzed using either a linear mixed-effect model or repeated-measures ANOVA. In the model, father ID was included as a random effect to adjust for dependence in results between offspring from the same father and litter size as a fixed variable to compare litter size variations between and within treatments and age as a fixed variable to determine whether there were any effects due to age.

Correlations of founder metabolic measurements (metabolites and adiposity) to founder sperm microRNA (\( \Delta \Delta C_T \)) were determined by a Pearson’s correlation or a partial correlation controlling for founder adiposity levels. Correlations of female offspring measurements to
Table 2. **Effect of diet and exercise on founder body composition, metabolism, and hormones**

<table>
<thead>
<tr>
<th></th>
<th>CC (n = 10)</th>
<th>HH (n = 11)</th>
<th>HC (n = 12)</th>
<th>HE (n = 10)</th>
<th>HCE (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preintervention</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total body weight</td>
<td>26.2 ± 1.4 a</td>
<td>31.5 ± 1.3 b</td>
<td>31.8 ± 1.2 b</td>
<td>30.8 ± 1.3 b</td>
<td>32.7 ± 1.3 b</td>
</tr>
<tr>
<td>Gonadal adiposity, %</td>
<td>2.46</td>
<td>2.36</td>
<td>2.68</td>
<td>2.68</td>
<td>2.70</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>3.12</td>
<td>3.07</td>
<td>3.13</td>
<td>3.17</td>
<td>3.16</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.24</td>
<td>0.23</td>
<td>0.25</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>12.2 ± 0.5</td>
<td>11.6 ± 0.5</td>
<td>11.3 ± 0.5</td>
<td>11.1 ± 0.5</td>
<td>12.3 ± 0.5 a</td>
</tr>
<tr>
<td><strong>Postintervention</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Total body weight</td>
<td>30.0 ± 1.15 b</td>
<td>36.2 ± 1.15 a</td>
<td>29.3 ± 1.15 a</td>
<td>31.9 ± 1.10 a</td>
<td>39.3 ± 1.10 a</td>
</tr>
<tr>
<td><strong>DEXA body composition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total adiposity, g</td>
<td>5.04 ± 0.70 a</td>
<td>5.04 ± 0.70 a</td>
<td>5.05 ± 0.69 a</td>
<td>7.26 ± 0.64 a</td>
<td>4.94 ± 0.65 a</td>
</tr>
<tr>
<td>Total adiposity, %</td>
<td>17.5 ± 1.66 b</td>
<td>28.5 ± 1.66 a</td>
<td>17.1 ± 1.65 a</td>
<td>22.9 ± 1.56 a</td>
<td>17.1 ± 1.55 a</td>
</tr>
<tr>
<td>Lean mass, g</td>
<td>23.2 ± 0.59 a</td>
<td>24.9 ± 0.58 a</td>
<td>23.1 ± 0.56 a</td>
<td>23.3 ± 0.54 b</td>
<td>23.3 ± 0.53 a</td>
</tr>
<tr>
<td>Bone, %</td>
<td>1.39 ± 0.04 a</td>
<td>1.16 ± 0.04 b</td>
<td>1.20 ± 0.03 b</td>
<td>1.21 ± 0.04 b</td>
<td>1.26 ± 0.03 b</td>
</tr>
<tr>
<td><strong>Postmortem body composition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose tolerance (GTT; AUC)</td>
<td>1.943 ± 101.6 b</td>
<td>2.089 ± 102.2 a</td>
<td>1.743 ± 97.8 a</td>
<td>1.607 ± 92.8 b</td>
<td>1.667 ± 98.6 a</td>
</tr>
<tr>
<td>Insulin tolerance (ITT; AUC)</td>
<td>148.2 ± 147.7 a</td>
<td>100.4 ± 146.4 b</td>
<td>136.7 ± 145.7 b</td>
<td>107.2 ± 140.8 b</td>
<td>126.5 ± 150.6 b</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>10.4 ± 0.41 b</td>
<td>10.9 ± 0.41 a</td>
<td>10.3 ± 0.39 b</td>
<td>8.98 ± 0.39 a</td>
<td>9.73 ± 0.39 b</td>
</tr>
<tr>
<td>Insulin, mg/ml</td>
<td>0.24 ± 0.17 b</td>
<td>0.21 ± 0.19 a</td>
<td>0.14 ± 0.18 b</td>
<td>0.43 ± 0.16 b</td>
<td>0.36 ± 0.20 b</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>3.12 ± 0.36 b</td>
<td>4.45 ± 0.4 b</td>
<td>3.00 ± 0.39 b</td>
<td>4.03 ± 0.34 b</td>
<td>2.25 ± 0.43 b</td>
</tr>
<tr>
<td>FF A, mg/ml</td>
<td>0.87 ± 0.05 b</td>
<td>0.92 ± 0.05 a</td>
<td>0.82 ± 0.05 b</td>
<td>0.86 ± 0.04 b</td>
<td>0.75 ± 0.05 b</td>
</tr>
<tr>
<td>Triglycerides, mg/ml</td>
<td>0.65 ± 0.05 a</td>
<td>0.67 ± 0.06 a</td>
<td>0.60 ± 0.06 a</td>
<td>0.68 ± 0.05 a</td>
<td>0.56 ± 0.06 a</td>
</tr>
<tr>
<td>Corticosterone, mg/ml</td>
<td>2.94 ± 1.20 a</td>
<td>1.18 ± 1.30 a</td>
<td>3.68 ± 1.28 a</td>
<td>5.07 ± 1.12 a</td>
<td>2.58 ± 1.41 a</td>
</tr>
<tr>
<td>CRP, mg/ml</td>
<td>398 ± 37</td>
<td>470 ± 29 a</td>
<td>538 ± 30 b</td>
<td>535 ± 28 c</td>
<td>493 ± 29 c</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. DEXA, dual-energy X-ray absorptiometry; CC, controls; HH, continuation of a HFD intervention group; HC, change to a CD intervention group; HE, continuation of a HFD with exercise intervention group; HCE, change to a CD with exercise intervention group; GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, area under the curve; AAC area above the curve; FFA, free fatty acids; CRP, C-reactive protein. Different letters denote significance at P < 0.05.

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spring (P < 0.05; Fig. 2D) and restored to that of CC offspring at both 10 and 18 wk (Fig. 2, D and G), concomitant with restoration of plasma adiponectin at 10 wk (Fig. 2E) to that of CC offspring, although by 18 wk plasma adiponectin concentrations were reduced compared with CC offspring (P < 0.05; Fig. 2E), similar to concentrations of HH offspring. Plasma cholesterol was not different at 10 wk (Table 4); however, it was reduced compared with CC female offspring at 18 wk (P < 0.05; Table 4), although it was not different from HH offspring, whereas no changes were seen in plasma triglyceride concentrations at 10 and 18 wk compared with HH and CC offspring (Fig. 2F). However, plasma FFA concentration was elevated at 18 wk compared with both CC and HH offspring (P < 0.05; Table 5). HC female offspring had normal glucose tolerance at 8 and 16 wk compared with both CC and HH offspring (Fig. 3, A–C), with improved insulin secretion at 16 wk compared with HH offspring maintaining their glucose levels during a glucose challenge (P < 0.05; Fig. 3, G and H). However, these offspring were hyperglycemic in the fasted state compared with CC offspring (P < 0.05; Table 5). HC females were insulin resistant at 9 wk of age compared with CC offspring (P < 0.05; Fig. 3, D and F), although at 17 wk they were not different from either CC or HH female offspring (Fig. 3, E and F). Interestingly, HC female offspring had
increases in corticosterone at 18 wk compared with CC offspring ($P < 0.05$; Table 5), which was not present at 10 wk (Table 4). These data imply that although there were some improvements to female offspring site-specific adiposity, adipocyte size, and health through diet interventions in their fathers, the residual impacts of founder HFD were still evident in the programming of the metabolic syndrome in female offspring.

Exercise-alone intervention (HE). FOUNDERS. Exercise intervention in founders normalized body weight and improved fasting plasma glucose ($15\%$) and glucose tolerance ($21\%$); however, it did not reduce their elevated adiposity ($24\%$) or cholesterol ($23\%$) compared with that of CC founders (Table 2). Additionally, HE founder plasma leptin and CRP were normalized to those of CC founders, whereas plasma corticosterone was numerically reduced but not differ-

### Table 3. Effect of diet and exercise on founder time to mate, mating rates, litter size, and sex ratio

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>HH</th>
<th>HC</th>
<th>HE</th>
<th>HCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to mate, days</td>
<td>2.44 ± 0.35</td>
<td>3.16 ± 0.40</td>
<td>2.91 ± 0.39</td>
<td>2.90 ± 0.35</td>
<td>3.14 ± 0.39</td>
</tr>
<tr>
<td>Successful mating, %</td>
<td>78.5$^a$$^b$</td>
<td>47.8$^a$</td>
<td>68.6$^a$</td>
<td>58.1$^a$</td>
<td>93.8$^a$</td>
</tr>
<tr>
<td>Litter size</td>
<td>20.0 ± 0.1</td>
<td>20.0 ± 0.1</td>
<td>19.8 ± 0.1</td>
<td>19.8 ± 0.1</td>
<td>19.9 ± 0.1</td>
</tr>
<tr>
<td>Female offspring, %</td>
<td>51$^a$$^b$</td>
<td>47$^a$</td>
<td>7.04 ± 0.24$^b$</td>
<td>6.32 ± 0.21$^a$</td>
<td>5.72 ± 0.22$^a$</td>
</tr>
</tbody>
</table>

Data are expressed per father and shown as means ± SE, and they were analyzed by a Fisher’s exact test. Data are representative of 8 HH, HC, HE, and HCE founder males and 7 CC founder males. Different letters denote significance at $P < 0.05$. Exercise-alone intervention (HE). FOUNDERS. Exercise intervention in founders normalized body weight and improved fasting plasma glucose ($15\%$) and glucose tolerance ($21\%$); however, it did not reduce their elevated adiposity ($24\%$) or cholesterol ($23\%$) compared with that of CC founders (Table 2). Additionally, HE founder plasma leptin and CRP were normalized to those of CC founders, whereas plasma corticosterone was numerically reduced but not differ-

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**Fig. 1.** Effect of diet and exercise as paternal obesity interventions on female offspring preweaning growth. A–C: preweaning growth of female offspring born from founder males undergoing diet alone intervention (HC; A), exercise alone intervention (HE; B), and combined diet and exercise intervention (HCE; C) compared with offspring born from controls (CC) and high-fat diet controls (HH). D: total amount of preweaning weight gained (day 5 until day 21). Data are represented as means ± SE. Different letters denote significance at $P < 0.05$. Data were analyzed using repeated-measures ANOVA, with father ID and mother ID added as a random factor and litter size as a fixed factor. Data represent at least 30 female offspring from 10 HH litters from 7 fathers, 14 HC litters from 7 fathers, 11 HE litters from 7 fathers, 19 HCE litters from 8 fathers, and 12 CC litters from 8 fathers.
ent from CC and HH founders (Table 2). HE did not alter time to mate, gestational length, litter size, or offspring sex ratio compared with CC founders (Table 3); however, it did reduce proportion of successful matings (−20%, P < 0.05; Table 3).

OFFSPRING. HE female offspring were similar in weight at postnatal days 5 and 7 compared with CC offspring (Fig. 1B) but were heavier by postnatal day 21 (P < 0.05; Fig. 1B), reflecting their increased weight gain preweaning compared with CC offspring (P < 0.05; Fig. 1D). HE offspring also had increased weight gain between 8 and 16 wk compared with HH offspring (P < 0.05; data not shown), although postweaning weight was not altered (data not shown). HE female offspring had increased renal fat depot at 10 wk compared with CC and HH offspring (P < 0.05; Fig. 2A), similar to HH offspring; however, by 18 wk these HE female offspring were not different from CC or HH female offspring (P < 0.05; Fig. 2A). These changes to adipose mass were mirrored by alterations in adipocyte cell size of gonadal fat, with increased cell size at 10 wk compared with CC offspring and HH offspring (P < 0.05; Fig. 2D), and reduced at 18 wk compared with HH offspring to a size similar to that of CC offspring (P < 0.05; Fig. 2D and G). Meanwhile, plasma adiponectin concentrations were restored to that of CC offspring at 10 wk (P < 0.05; Fig. 2E) and further elevated at 18 wk (P < 0.05; Fig. 2E). HE female offspring had partial restoration of plasma triglycerides at 8 wk with concentrations not different from either CC or HH offspring (Fig. 2F). By 18 wk plasma triglycerides were reduced compared with HH offspring (P < 0.05; Fig. 2F), restoring levels to that of CC offspring. HE offspring had reduced fasting plasma glucose at 10 wk compared with CC and HH offspring (P < 0.05; Table 4). Consistent with this, HE offspring had improved glucose tolerance, as evidenced by a reduced AUC at 8 wk compared with CC and HH female offspring (P < 0.05; Fig. 3, A and C), with restoration of insulin secretion during a glucose challenge at both 8 and 16 wk compared with HH offspring (P < 0.05; Fig. 3, G–J). HE offspring had restoration of insulin sensitivity, as evidenced by a larger AAC postinsulin challenge compared with HH and CC offspring at both 9 and 17 wk of age (Fig. 3, D–F). Similar to both HC and HH female offspring, HE female offspring had increased plasma corticosterone at 18 wk compared with CC offspring (P < 0.05, Table 5) that was not present at 10 wk of age (Table 4). These data imply that exercise intervention alone in obese males may reduce female offspring susceptibility to metabolic syndrome.

Combined diet/exercise intervention (HCE). FOUNDERS. Combined diet/exercise interventions in obese founders reduced weight (−23%), adiposity (−42%), plasma cholesterol (−49%), leptin (−78%), CRP (−21%), fasting plasma glucose (−10%), and FFA (−18%) compared with HH (P < 0.01; Table 2). Additionally, improved glucose tolerance (+17%) was observed compared with both HH and control founders.

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(P < 0.05; Table 2). However, plasma corticosterone remained elevated compared with CC founders (+20%), similar to that of HH founders (Table 2). There was no effect of HCE on time to mate or gestational length between CC and HH founders (Table 3); however, HCE restored successful matings (+46%) compared with HH founders to levels similar to CC founders (P < 0.05; Table 3). Interestingly, HCE founders had reduced litter sizes compared with HH founders (−9%, P < 0.05; Table 3), although this resulted in a difference of only 0.5 pups per litter and was not different from CC founders (Table 3). HCE founders had similar sex ratios of offspring to that of CC and HH founders, although the proportion of female offspring was reduced compared with the other founder intervention groups (HC and HE, P < 0.05; Table 3), indicating that HCE founders may have an increased proportion of X:Y chromosomes bearing sperm or differential survival of female embryos or fetuses (13).

OFFSPRING. HCE female offspring were of similar weights at postnatal days 5 and 7 compared with CC and HH offspring (Fig. 1C) but were heavier by postnatal day 12 onward compared with CC offspring (P < 0.05; Fig. 1C), which was reflected by their increased weight gain preweaning compared with both CC and HH offspring (P < 0.05; Fig. 1D). This increase in growth rate continued up until 8 wk of age (P < 0.05; data not shown), although total body weight was not different from CC and HH offspring at each weekly time point (data not shown), nor was weight gain different between 8 and 16 wk of age (data not shown). At 10 wk, HCE offspring had reduced total adiposity, as measured by DEXA compared with HH offspring (P < 0.05; Table 4), which was not different from CC offspring, with a similar restoration to that of CC offspring seen for renal adipose depot at 18 wk (Fig. 2A). These changes to adipose mass were mirrored by alterations in adipocyte cell size of gonadal fat, with normalization of adipocyte size evident at 10 wk compared with HH offspring (P < 0.05; Fig. 2E) and CC offspring. Although adipocyte cell size was still reduced at 18 wk compared with HH offspring (P < 0.05; Fig. 2, D and G), it was larger than that of CC offspring (P < 0.05; Fig. 2, D and G). HCE offspring plasma adiponectin concentrations were restored to that of CC offspring at both 10 and 18 wk of age (Fig. 2E) but were greater than those of HH offspring (P < 0.05, Fig. 2E). At 10 wk of age, HCE offspring had increased plasma cholesterol compared with both CC and HH offspring (P < 0.05, Table 4) that was no longer evident at 18 wk (Table 5). Additionally, at 18 wk, HCE offspring had increased plasma triglycerides compared with CC offspring (P < 0.05; Fig. 2F), similar to HH offspring concentration, which coincided with increased liver weights both as absolute mass and proportion of total body weight compared with both CC and HH offspring (P < 0.05; Table 5). HCE offspring had normal glucose tolerance at 8 and 16 wk of age compared with CC and HH offspring (Fig. 3, A–C). Insulin sensitivity was improved compared with HH offspring at 9 wk (P < 0.05; Fig. 3, D and F) and restored to that of CC offspring. However, this improvement was not maintained, and by 17 wk HCE offspring were insulin resistant compared with CC offspring (P < 0.05;
However, founder adiposity positively correlated with female offspring (Table 2) but showed the greatest restoration of phenotypic liability to metabolic disease. In founders were not able to fully reprogram offspring susceptibility relative to the exercise-only intervention may suggest that the combined diet and exercise approach and changes associated with this and/or the accelerated rate of weight loss in founders were not able to fully reprogram offspring susceptibility to metabolic disease.

Paternal Metabolic and Hormonal Factors and Offspring Phenotype

As HE founders maintained their preintervention level of adiposity (Table 2) but showed the greatest restoration of female offspring metabolic phenotypes, we determined whether other founder metabolic markers independent of adiposity correlated with female offspring phenotypes. There was no correlation between founder adiposity or preweaning weight, postweaning weight, or glucose and insulin tolerance. However, founder adiposity positively correlated with female offspring renal adipose depot (%total body weight) at 10 wk (0.337, \( P = 0.02 \)) and 18 wk (0.350, \( P = 0.02 \)), omental adipose depot (%total body weight) at 10 (0.285, \( P = 0.04 \)) and 18 wk (0.334, \( P = 0.03 \), and summed adipose depot (% total body weight) at 18 wk (0.312, \( P = 0.04 \)) while negatively correlating with female offspring plasma adiponectin at 18 wk (−0.457, \( P = 0.05 \)).

We further determined whether other founder metabolic markers also correlated with offspring phenotypes and whether these metabolic markers were dependent on founder adiposity. Founder corticosterone, which was increased in HH founders and only partially restored in HE founders (Table 2), correlated negatively with female offspring preweaning size (\( P < 0.05 \); Table 6) and positively with renal and omental adiposity at 10 wk (\( P < 0.05 \); Table 6). The positive association with founder corticosterone and offspring renal adiposity was still evident even after controlling for paternal adiposity (0.320, \( P = 0.04 \)). Additionally to founder corticosterone, founder plasma cholesterol, which was restored to that of CC founders in those males undergoing diet intervention (HC and HCE) (Table 2), also correlated with offspring preweaning size, but in a positive manner (\( P < 0.05 \); Table 2). Founder plasma cholesterol also correlated positively with offspring adiposity at 10 (\( P < 0.05 \); Table 6) and 18 wk (\( P < 0.05 \); Table 6), with correlations to summed adipose depot at 10 and 18 wk remaining after controlling for paternal adiposity (0.349, \( P = 0.03 \), and 0.374, \( P = 0.01 \), respectively). Founder triglycerides, which were not altered by founder diet or intervention (Table 2), correlated positively with offspring day 21 weights (\( P < 0.05 \); Table 6).

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**Table 5. Effect of founder diet and exercise interventions on F1 female body composition, metabolites, and hormones at 18 wk of age**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>CC</th>
<th>HH</th>
<th>HC</th>
<th>HE</th>
<th>HCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight</td>
<td>19.8 ± 0.12^b</td>
<td>20.0 ± 0.19^a</td>
<td>19.4 ± 0.18^b</td>
<td>19.3 ± 0.18^b</td>
<td>19.8 ± 0.19^a,b</td>
</tr>
<tr>
<td>Dorsal adiposity, %</td>
<td>0.40 ± 0.02^a</td>
<td>0.43 ± 0.03^b</td>
<td>0.37 ± 0.02^a</td>
<td>0.38 ± 0.03^a</td>
<td>0.42 ± 0.02^a</td>
</tr>
<tr>
<td>Sum of adiposity depots, %</td>
<td>1.44 ± 0.13^a</td>
<td>1.53 ± 0.13^a</td>
<td>1.37 ± 0.13^a</td>
<td>1.40 ± 0.12^a</td>
<td>1.39 ± 0.12^a</td>
</tr>
<tr>
<td>Liver, g</td>
<td>0.75 ± 0.03^a</td>
<td>0.74 ± 0.03^a</td>
<td>0.70 ± 0.02^a</td>
<td>0.78 ± 0.02^ab</td>
<td>0.83 ± 0.02^b</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>3.78 ± 0.12^ae</td>
<td>3.78 ± 0.13^a</td>
<td>3.62 ± 0.12^a</td>
<td>4.00 ± 0.13^ae</td>
<td>4.19 ± 0.13^a</td>
</tr>
<tr>
<td>Kidneys, %</td>
<td>0.12 ± 0.01^a</td>
<td>0.14 ± 0.01^a</td>
<td>0.14 ± 0.00^a</td>
<td>0.13 ± 0.01^a</td>
<td>0.13 ± 0.01^a</td>
</tr>
<tr>
<td>Kidneys, g</td>
<td>0.09 ± 0.06^a</td>
<td>0.10 ± 0.05^b</td>
<td>0.10 ± 0.04^b</td>
<td>1.06 ± 0.04^a</td>
<td>1.04 ± 0.03^ab</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>7.72 ± 0.65^a</td>
<td>7.82 ± 0.68^a</td>
<td>7.26 ± 0.76</td>
<td>7.67 ± 0.64^a</td>
<td>7.84 ± 0.36^a</td>
</tr>
<tr>
<td>Vastus lateralis, mg</td>
<td>115.9 ± 3.6^ah</td>
<td>120.9 ± 5.4^a</td>
<td>101.9 ± 2.6^a</td>
<td>120.7 ± 4.0^a</td>
<td>108.2 ± 3.8^ac</td>
</tr>
</tbody>
</table>

**Metabolites and hormones**

- Glucose, mmol/l: 8.18 ± 0.22^a, 8.41 ± 0.23^ab, 8.90 ± 0.22^b, 8.46 ± 0.22^ab, 8.45 ± 0.21^ab
- Cholesterol, mmol/l: 1.92 ± 0.10^a, 1.72 ± 0.11^ab, 1.66 ± 0.09^b, 1.83 ± 0.08^ab, 1.96 ± 0.09^b
- FFa, mmol/l: 0.57 ± 0.05^ac, 0.64 ± 0.05^c, 0.82 ± 0.05^c, 0.55 ± 0.05^c, 0.69 ± 0.05^c
- Insulin, ng/ml: 0.24 ± 0.09^a, 0.12 ± 0.10^a, 0.20 ± 0.09^a, 0.12 ± 0.08^a, 0.10 ± 0.07^a
- Leptin, mg/ml: 0.49 ± 0.22^a, 0.61 ± 0.16^b, 0.52 ± 0.18^b, 0.53 ± 0.18^b, 0.52 ± 0.20^a
- Corticosterone, ng/ml: 537 ± 106^a, 685 ± 110^a, 849 ± 101^b, 856 ± 87^a, 864 ± 89^b

Data are expressed as means ± SE. DEXA data represent 10 females from 10 litters representing \( n = 7 \) HH, HC, HE, HCE, and CC males. Postmortem and metabolite data represent 7 females from 7 litters representing \( n = 6 \) HH, HC, HCE, and CC males and 8 females from 8 litters representing \( n = 5 \) HE males. Data were matched for litter size and analyzed using either repeated-measures ANOVA or linear mixed-effects model with father ID as a random effect and litter size and age as fixed effects. Different letters denote significance at \( P < 0.05 \). Total body weight was measured at postmortem. DEXA data were analyzed to body weight recorded at DEXA.

Fig. 3, E and F), similar to HH offspring. At this same age, HCE female offspring secreted more insulin compared with CC offspring, similar to that of HH offspring to maintain normal glucose tolerance seen at the both the 8- and 16-wk GTT (\( P < 0.05 \); Fig. 3, G–J). Similar to both HC and HE female offspring, HCE female offspring also had increased fasting plasma corticosterone at 18 wk compared with CC offspring (\( P < 0.05 \); Table 5) that was not elevated at 10 wk (Table 4). These data imply that combined diet and exercise interventions in the father can restore adipose health in female offspring. However, the incremental improvements to insulin sensitivity relative to the exercise-only intervention may suggest that the combined diet and exercise approach and changes associated with this and/or the accelerated rate of weight loss in founders were not able to fully reprogram offspring susceptibility to metabolic disease.
Fig. 3. Effects of diet and exercise on diet-induced paternal obesity on insulin sensitivity, glucose tolerance, and insulin secretion in female offspring. A and B: glucose tolerance as assessed by glucose tolerance test (GTT; 2 g/kg) at 8 (A) and 16 wk (B) of age. C: glucose area under the curve (AUC; mmol/min) during GTT over age. D and E: insulin sensitivity as assessed by insulin tolerance test (ITT; 0.75 IU) at 9 (D) and 17 wk (E) of age. F: insulin sensitivity [glucose area during ITT, area above the curve (AAC), mmol/min] with age. G: insulin secretion during GTT at 16 wk. H: insulin secretion AUC during GTT at 16 wk. J: insulin secretion relative to GTT (ng·ml⁻¹·glucose mmol⁻¹·l⁻¹ insulin at 0, 15, 30, and 60 min during the GTT) at 16 wk. Data are represented as means ± SE. Different letters denote significance at \( P < 0.05 \). Data were matched for litter size and analyzed using repeated-measures ANOVA or linear mixed-effects modeling, with father ID added as a random factor and litter size as a fixed effect. ITT and GTT data represent 10 females from 10 litters representing 7 males/treatment group. Insulin secretion during a GTT represents 7 females from 7 litters representing 4 males/treatment group.
and renal adiposity depots at 10 wk ($P < 0.05$; Table 6) while negatively correlating with offspring glucose tolerance at 8 wk ($P < 0.05$; Table 6). However, the correlation between founder plasma triglyceride concentration and offspring renal adiposity was found once it was controlled for founder adiposity. Founder plasma leptin and plasma CRP, which were reduced in all interventions compared with HH founders (Table 2), also correlated positively with offspring summed adipose depots at 18 wk ($P < 0.05$; Table 6), with correlations to summed adipose depots at 18 wk remaining evident after controlling for paternal adiposity ($0.389$, $P < 0.01$).

Paternal glucose clearance, which was improved in all founder interventions compared with HH founders (Table 2), correlated with metabolic measurements in offspring (Table 6).
Founder fasted plasma glucose levels correlated positively with offspring total body weight at 21 days and 18 wk ($P < 0.05$; Table 6) and glucose tolerance (AUC) at 8 wk ($P < 0.05$; Table 6) while correlating negatively with offspring omental adiposity depot at 10 wk ($P < 0.05$; Table 6) and glucose tolerance (AUC) at 16 wk ($P < 0.05$; Table 6). Founder glucose concentration negative correlation with omental adiposity depot at 10 wk and founder glucose tolerance negative correlation with renal adiposity depot at 10 wk persisted after controlling for paternal adiposity ($-0.539$, $P < 0.01$, and $-0.370$, $P = 0.03$, respectively). Additionally, paternal fasted FFA concentrations, which were reduced in HCE founders compared with HH founders (Table 2), positively correlated with offspring fasted FFA concentrations at 10 wk ($P < 0.05$; Table 6) and negatively with offspring adipose depots at 18 wk ($P < 0.05$; Table 6), with all correlations remaining significant after controlling for paternal adiposity (offspring summed adipose depots $-0.364$, $P = 0.02$). This implicates paternal systematic metabolic health as a potential mediator of offspring phenotypes independent of paternal adiposity.

Paternal Epigenetic Signals: Sperm MicroRNA Profiles

Any improvements to offspring health transmitted through the father may be delivered to the developing embryo through sperm at fertilization. Sperm deliver a vast array of RNAs, including microRNAs, to the oocyte at fertilization that can influence first cleavage stage, embryo development, and the phenotype of subsequent offspring (28). We decided to prioritize X-linked sperm microRNAs (data not shown) from the original HFD microRNA array card data (18) due to the transmission pattern of the most acute metabolic phenotypes seen in our original study (grandfather to daughter to grandson) (18) and the improvements to female offspring seen in the current study. Eight X-linked sperm microRNAs in 10-wk HFD-challenged founders were found to be differentially abundant compared with controls ($P < 0.05$, fold change $\geq 1.5$; data not shown). We then confirmed that these eight X-linked sperm microRNAs were also differentially abundant in CC and HH in founders in our current study and that a HFD followed by diet and/or exercise interventions further modulated their abundance. The abundance of 4 X-linked sperm microRNAs (mir-503, mir-456b-5p, mir-542-3p, and mir-652) were confirmed to be altered in our HH vs. our CC founders (albeit in the opposite direction with mir-542-3p and mir-456-5p, $P < 0.05$; Fig. 4).

Of the four X-linked sperm microRNAs altered in HH founders compared with CC founders (mir-503, mir-456b-5p, mir-542-3p, and mir-652, $P < 0.05$; Fig. 4), three of them were restored by diet and/or exercise interventions (mir-503, mir-465b-5p, and mir-542-3p; Fig. 4). Interestingly, HE founders still displayed reduced mir-503 abundance in sperm compared with CC founders ($P < 0.05$; Fig. 4B), similar to HH founders. Mir-465b-5p abundance in sperm was restored in all diet intervention groups (HC and HCE) displaying levels similar to CC founders (Fig. 4C), whereas HE founders’ sperm mir-456b-5p abundance was increased compared with all other founder groups (including CC, $P < 0.05$; Fig. 4C). Mir-542-3p abundance was only restored in HCE founders the same as CC founders and less abundant than HH founders ($P < 0.05$; Fig. 4D). Interestingly, HC and HE founders had increased abundance of sperm mir-542-3p, exceeding levels of both HH and CC founders ($P < 0.05$; Fig. 4D). Mir-652 was not restored by diet and/or exercise interventions, with abundance further reduced in all founder groups compared with HH and CC ($P < 0.05$; Fig. 4E).

The remaining four X-linked sperm microRNAs (mir-883a-5p, mir-465a-3p, mir-871, and mir-743b-3p) were not altered in HH founders compared with CC founders, but interestingly, they were altered by diet and/or exercise intervention (HC, HE, and HCE; data not shown). Mir-883a-5p abundance was up-regulated in HC (+4.0-fold) and HE (+3.9-fold) founders compared with both CC and HH founders ($P < 0.05$; data not shown), whereas HCE founders’ abundance was not different. Mir-465a-3p abundance was increased in HC founders compared with both CC and HH founders (+3.3- and +2.4-fold, respectively, $P < 0.05$; data not shown), whereas HE abundance was only increased compared with CC founders (+1.8-fold, $P < 0.05$; data not shown), with levels similar to HH founders. There was no change in mir-465a-3p abundance in HCE founders, with levels similar to both CC and HH founders (data not shown). Mir-871 abundance was increased in HCE founders compared with CC founders (+2.1-fold, $P < 0.05$; data not shown), with levels similar to HH founders. HC and HE founders displayed no change in mir-871 abundance, with levels similar to both CC and HH founders (data not shown). Mir-743b-3p abundance was increased in all interventions compared with CC founders (HC +2.3-fold, HE +2.5-fold, and HCE +2.0-fold, $P < 0.05$; data not shown), whereas HE founders’ abundance was also increased compared with HH founders (+2.3-fold, $P < 0.05$; data not shown). These data suggest that sperm microRNA profile is sensitive not only to diet but also to changes in diet and/or exercise.

Paternal Glucose Regulation and Sperm microRNA

Similarly to offspring phenotypes, we further determined whether other metabolic markers other than adiposity correlated with sperm microRNA abundance. We first assessed those microRNAs that were altered in abundance between HH and CC founders but restored by diet and exercise interventions (mir-503, mir-456b-5p, mir-542-3p, and mir-652). Founder adiposity correlated positively with sperm mir-542-3p abundance (0.348, $P = 0.04$), which had increased abundance in sperm from HH founders (Fig. 4D). The only founder metabolic measure beside adiposity that correlated with sperm X-linked microRNAs was glucose regulation. Founder fasted plasma glucose levels also correlated with sperm mir-542-3p abundance, albeit in a negative direction ($P < 0.01$; Table 6). When controlling for paternal adiposity, this negative correlation between founder fasted plasma glucose levels and sperm mir-542-3p remained significant ($-0.412$, $P = 0.02$). Additionally, founder glucose tolerance (GTT, AUC) correlated positively with sperm mir-532-3p abundance ($P < 0.05$; Table 6).

We then determined whether paternal fasting glucose or glucose tolerance (GTT, AUC) correlated with those sperm microRNAs that had differential abundance due to diet and/or exercise interventions but not HH or CC founder sperm (mir-871, mir-465a-3p, mir-743b-3p, and mir-883a-5p; data not
shown). Founder fasting plasma glucose correlated positively with sperm mir-871 abundance ($P < 0.05$; Table 6), whereas founder glucose tolerance (GTT, AUC) correlated positively with sperm mir-871 abundance ($P < 0.05$; Table 6), sperm mir-465a-3p abundance ($P < 0.05$; Table 6), mir-743b-3p abundance ($P < 0.05$; Table 6), and mir-883a-5p abundance ($P < 0.05$; Table 6). This implicates paternal systematic glucose regulation as a potential mediator in sperm X-linked microRNA abundance. Interestingly, both founder fasted plasma glucose and glucose tolerance were the most improved in interventions involving exercise (HE and HCE) compared with CC and HH founders ($P < 0.05$; Table 2), with HE and HCE founders additionally showing the biggest restorations of X-linked sperm microRNA profiles (Fig. 4).

DISCUSSION

Paternal obesity at conception has previously been shown to induce metabolic syndrome through two generations, with an earlier onset and worsened phenotype in female offspring (17, 45). Therefore, we examined whether short-term (8 wk, 2 rounds of spermatogenesis) diet and exercise interventions in the father could improve this altered metabolic phenotype of female offspring and determine any other contributing factors independent of adiposity that could be mediating these effects.

Prolonged Paternal HFD Feeding Induced Insulin Resistance and Increased Adipose Accumulation in Female Offspring

Similar to previous studies (18, 45), a paternal HFD that induced obesity prior to conception resulted in insulin resistance, impaired insulin secretion, and increased adipose accumulation in female offspring. Extending these studies, we have found that female offspring of HFD founders had an altered adipocyte phenotype (size and secretory profile) prior to the onset of overt obesity. This observation is consistent with the recent observation whereby paternal HFD changed the transcriptome of genes in pathways regulating cellular response to stress, telomerase signaling, cell death, cell cycle, and cell growth in white adipose tissue of female offspring in the absence of obesity (46). Because C57BL6 mice are prone to metabolic disease (62), this suggests an increased, rapid deterioration of female offspring as a result of aging from founder HFD feeding.

Short-Term Founder Diet/Exercise Intervention Restored Adipocyte Size and Insulin Resistance in Female Offspring

Diet intervention (HC and HCE) was the only intervention treatment to improve the previously reported reduced mating rates of HFD-fed males (20). Interestingly, in humans, an overweight or obese male partner with a female of normal body mass index has an increased-odds ratio for time to conceive (47, 59) and increased rates of sexual dysfunction (55). This reported sexual dysfunction in humans has been shown to be reversed by weight loss through diet and exercise interventions (24, 40). Because our exercise-only group (HE) maintained their preintervention adiposity and reduced mating rates, mating success maybe more related to adiposity and the hormonal changes that can be associated with this (12, 29, 63).

All combinations of interventions in obese founder males resulted in some measure of restoration to the metabolic health of their female offspring, however surprisingly; the biggest improvements to offspring phenotypes (adipocyte size and insulin regulation) resulted from the founder exercise-only intervention despite maintained increased adiposity (HE). This suggests that the positive benefits of exercise may override some of the negative effects of increased adiposity. Single bouts of short-term exercise before consumption of a high-fat meal in obese adolescents reduce circulating lipids (34). Interestingly, founder plasma lipids correlated with offspring phenotypes independently of adiposity in this study. Increased serum cholesterol without marked increases to body weight in a rabbit model has previously been reported to cause sperm dysfunction with reduced sperm motility, count, morphology, capacitation, and semen volume (64). Additionally, exposure to increasing in vitro concentrations of both cholesterol and FFA in human sperm caused increased levels of oxidative stress (30), with chemically induced increases in sperm oxidative stress previously reported to program obesity and metabolic syndrome in female offspring (32).

Although exercise-only (HE) founders had increased adiposity compared with controls, the function of their adipose tissue was likely altered due to beneficial physiological outcomes from exercise. This phenomenon has been reported previously in a human study of diet and exercise interventions in obese adolescents with males who underwent exercise interventions without any alterations to adiposity showing improvements to inflammatory pathways in their adipose tissues (4). To this end, exercise intervention alone (HE) restored circulating CRP concentrations, thus reducing the proinflammatory state that was associated with males fed a high-fat diet (HH). It has becoming increasingly apparent that metabolic disease and obesity are associated with low-grade chronic inflammation (25). A proinflammatory state induced by infection, environmental toxins, smoking, and vasectomy reversals in men is associated with subfertility. The evidence for this subfertility is shown by reduced sperm counts, sperm motility, and sperm morphology, increased anti-sperm antibodies, increased sperm reactive oxygen species, and DNA damage (6), which bare striking similarities to the subfertility phenotypes seen in obese men (52). Additionally, some paternal proinflammatory conditions, including smoking and environmental toxin exposure, are also associated with fathering children with increased susceptibility to cancer and childhood malformations (11, 67). However, whether offspring health pathologies are programmed directly as a result of the proinflammatory state or another paternal phenotype that results from these conditions remains to be determined.

Fig. 4. Effect of diet and exercise interventions in diet-induced paternal obesity on X-linked sperm microRNAs. A: chromosomal localization of X-linked mouse microRNAs adapted from (56); note that microRNAs in boldface are localized in homologous regions on the human X chromosome. B: fold change abundance of mir-503 (B), mir-465a-3p (C), mir-542-3p (D), and mir-652 (E) in founder sperm expressed as fold change relative to CC founders. Data are represented as means ± SE. Different letters denote significance at $P < 0.05$. Data were analyzed by 1-way ANOVA. Data represent $n = 6$ HH and CC founders and $n = 8$ HC, HE, and HCE founders.
It has been reported previously that sperm functions, specifically sperm DNA damage and reactive oxygen species, are fully restored by exercise-only intervention in founder males (53), with these measures themselves independently related to reduced blastocyst quality and compromised pregnancy outcomes (1, 5, 71). Whether the improved female offspring health seen here as a result of the relatively brief intervention window in their father (8 wk in this study) could be further improved by an intervention of longer duration also remains to be determined. Additionally, one of the original rodent models determined that feeding a high-fat diet to induce obesity in male mice perturbed the metabolic health across two generations (18). Therefore, the improvements of the F1 female offspring phenotype seen in this study may cascade to F2 offspring, also restoring metabolic health through the pathway of multigenerational transmission of metabolic consequences initiated by grand paternal obesity.

Although diet interventions (HC and HCE) did display some restoration of offspring phenotypes, not all metabolic measurements were improved to the extent of the HE intervention, indicating that some residual impacts of the HFD remained, such as the previously increased adiposity or the influence of a change to a diet. These fathers displayed significant weight loss, and whether the nonoptimal improvements to the health of their offspring relates to a stressed state due to rapid weight loss, thereby increasing free radicals in sperm, remains unknown. However, plasma corticosterone, a marker of stress that was still elevated in these two groups compared with HH founders, did correlate with a number of offspring phenotypes, including preweaning growth. It has been shown previously that either a chronic or acute paternal stressor prior to mating is associated with altered offspring phenotypes, including reduced postnatal growth in female offspring (26) and changes to hypothalamic–pituitary–gonadal axis stress responsively (61). Together, these data implicate obesity-induced stress as a mediator for paternal programming.

X-linked Sperm MicroRNAs as Molecular Mediators of Paternal Programming

The detrimental effects to female offspring metabolic health due to paternal obesity (18, 45) suggest that a molecular mechanism is being transmitted via sperm. One such proposed mechanism underlying paternal programming is the transmission of noncoding RNAs. Mature spermatozoa contain significant amounts of RNA and noncoding RNAs, including microRNAs (51). MicroRNAs are best described as mediators of mRNA decay, and during fertilization sperm RNA content is delivered to the oocyte (51). Altered microRNA content in the male pronucleus has previously been shown to precipitate offspring phenotypes. Microinjection of a single microRNA or its predicted target mRNA into the male pronucleus of mouse embryos can alter resultant offspring phenotypes, including loss of pigmentation (60), cardiac hypertrophy (68), and increased growth trajectories (21). There already exists evidence that the microRNA composition of spermatozoa can respond to environmental factors such as stress and via this mechanism make an important epigenetic contribution to the progeny that persists into future generations (19). Previously, we have reported dysregulation of microRNAs in the testes and mature spermatozoa of rodent males fed a high-fat diet. Furthermore, this altered levels of mRNA targets involved in molecular networks that are involved in embryonic development (pluripotency), metabolic disease (leptin/insulin signaling and carbohydrate/lipid metabolism), transcriptional regulation, RNA posttranslational modification, and inflammation (18), consistent with embryo and offspring phenotypes observed from this model. Furthermore, altered microRNA content in serum of obese men can be restored by diet and exercise (50). Therefore, it is plausible that sperm microRNAs may be similarly restored by diet and exercise, and therefore, they may provide a partial explanation for the improvements observed in embryo and offspring health as a result of diet and/or exercise interventions in obese males.

We focused specifically on X-linked microRNAs in this study due to the heightened female offspring metabolic pathologies reported in the original studies and the fact that the majority of X-linked microRNAs in sperm escape meiotic sex chromosome inactivation during spermatogenesis (66). MiR-503 abundance was restored in sperm from founders undergoing exercise intervention (HE and HCE; Fig. 4A). MiR-503 has experimentally confirmed mRNA targets in the cyclin family that are involved in cell cycle regulation, which is critical for G1 to S phase progression. Cyclins are required after fertilization for oocyte meiotic resumption and first-stage cleavage events in the developing embryo (70). MiR-542-3p targets BIRC5, an inhibitor of apoptosis that when upregulated in zebra fish embryos increases apoptosis in the inner cell mass (36). MiR-542-3p was restored only in those founders undergoing combined diet and exercise interventions, which is consistent with the restoration of inner cell mass cell numbers in developing embryos sired by them (38). Although mir-465b-5p has currently no confirmed gene targets, it is expressed abundantly in neonatal ovaries, suggesting that it might have a role in female fertility. Mir-465b-5p abundance was restored in founders undergoing diet intervention (HC and HCE) and upregulated in exercise intervention alone. Given the likely impact of X-linked microRNAs on early embryo development, it may be possible that male and female embryos are affected differently due to the possible dosage effects between females (2 X chromosomes) and males (1 X chromosome) and the fact that X chromosome inactivation does not occur until the blastocyst stage embryo, explaining why we see a bigger impact of paternal programming in female offspring.

Although all intervention groups showed some degree of sperm microRNA normalisation, exercise interventions (HE and HCE) typically showed the greatest improvements. The mechanism by which exercise restores these microRNAs remains to be investigated. However, it has been shown in humans that even short bouts of exercise can alter the microRNA profile of serum (8, 65). It should be noted that exercise interventions in founders (HE and HCE) were previously shown to result in the greatest improvements to embryo development (38) and insulin sensitivity in female offspring at 9 wk of age in this study. However, the improvements to offspring metabolism were not always consistent across ages assessed (i.e., HCE), suggesting that the mechanism is clearly multifactorial, with restoration of sperm microRNAs likely only part of a broader epigenetic and genetic landscape altered in sperm due to obesity.

MicroRNAs can be regulated by an individual’s metabolic phenotype specifically playing important roles in the regulation
of glucose homeostasis (14). Therefore, it is likely that the differentially abundant microRNAs that result from diet and/or exercise interventions were likely due to improved glucose tolerance. This idea is supported by the correlation between glucose tolerance and X-linked sperm microRNA abundance independently of adiposity. Interestingly, serum glucose levels in humans have been shown previously to correlate with serum microRNA levels (57); however, whether changes to glucose concentrations alter microRNA abundance or vice versa remains to be elucidated. Additionally, elevated glucose concentrations can impair the molecular makeup of sperm, resulting in increased sperm DNA damage and increased reactive oxygen species in men with type 1 or type 2 diabetes (2, 31, 58). Furthermore, diabetic fathers are known to program metabolic phenotypes in offspring in both human and animal settings (3, 22, 39, 69).

Conclusion

Our findings further extend the concept of developmental programming of adult disease to include a paternal role in the early life origins of disease. We have identified a novel intervention window for the improvement of adverse offspring phenotypes through interventions in the obese father, providing a potential circuit breaker for the generational amplification of the obesity/metabolic syndrome. Our study provides some of the first evidence that lifestyle interventions that address paternal obesity can normalize sperm microRNA abundance. These microRNAs are known to target genes necessary for successful embryogenesis, potentially mediating the partial normalization of female offspring predisposition to obesity and metabolic disease that results from these interventions. Thus, implying environmental exposure throughout spermatogenesis can alter signals in sperm that program/reprogram female offspring phenotype. Furthermore, we have identified paternal glycemia, inflammation, plasma lipids, and stress as potential mediators for offspring programming, providing unrecognized targets for future interventions to compliment/replace diet and exercise. Future studies directed at identifying critical periods of paternal environmental exposure during spermatogenesis and the key metabolic signals and mediating factor(s) within the testes, thus identifying an as-yet unrecognized intervention target to prevent/overcome adverse paternal developmental programming, are warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.O.M., J.A.O., T.F., and M.L. conception and design of research; N.O.M. performed experiments; N.O.M. and M.L. analyzed data; N.O.M., J.A.O., T.F., and M.L. interpreted results of experiments; N.O.M. prepared figures; N.O.M. drafted manuscript; N.O.M., J.A.O., T.F., and M.L. edited and revised manuscript; N.O.M., J.A.O., T.F., and M.L. approved final version of manuscript.

REFERENCES


