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Fathers That Are Born Small Program Alterations in the Next-Generation Preimplantation Rat Embryos[1,2]

Jordanna S Master,[3] George A Thouas,[4] Alexandra J Harvey,[4] John R Sheedy,[4] Natalie J Hannan,[4,5] David K Gardner,[4] and Mary E Wlodek[3]*

Departments of [3]Physiology and [4]Zoology, The University of Melbourne, Parkville, Australia; and [5]Department of Obstetrics and Gynaecology, The University of Melbourne, Mercy Hospital, Heidelberg, Australia

Abstract

Background: Low birth weight is associated with increased risk of adult cardiovascular and metabolic disease development, with recent studies highlighting transmission to subsequent generations via both maternal and paternal lines. However, the timing of parent-specific programming of disease risk to the next generation remains to be characterized.

Objective: The aim of this study was to examine how paternal low birth weight affects the cellular and molecular physiology of the next-generation [second-generation (F2)] blastocysts, before uterine implantation.

Methods: Uteroplacental insufficiency was surgically induced in Wistar Kyoto pregnant rats in late gestation, giving rise to first-generation restricted (born small) and sham-operated control (normal birth weight) male offspring, respectively. First-generation restricted and control male rats were naturally mated with normal females.

Results: Resultant F2 blastocysts derived from restricted males displayed reduced expression of growth regulatory genes of the mammalian target of rapamycin pathway compared with F2 control blastocysts (9–74%; $P < 0.05$). No differences were found in F2 restricted blastocyst structural characteristics, cell number, or carbohydrate utilization at the time of blastocyst retrieval or after 24 h of in vitro culture. However, histidine, methionine, pyruvate, serine, and tryosine consumption and aspartate and leucine production were greater in F2 restricted outgrowth than in controls ($P < 0.05$).

Conclusions: The findings from this study clearly indicate that male rat offspring born small, arising from uteroplacental insufficiency, have physiologic alterations that manifest as modifications in gene expression levels and nutrient metabolism of F2 blastocysts, even in the absence of overt cellular growth differences. These data demonstrate that growth restriction and associated disease risk have the capacity to be transmitted to the next generation of offspring via the male germ line and is manifest as early as the blastocyst stage of development. *J Nutr* 2015;145:876–83.

Keywords: preimplantation rat blastocyst, uteroplacental insufficiency, transgenerational transmission, growth restriction, paternal line

Introduction

Intrauterine growth restriction, characterized by birth weight below the 10th percentile for gestational age, is commonly caused by placental dysfunction in the Western world (1–3). Epidemiologic studies have highlighted that being born small is linked to increased disease risk, not only for that individual but also for subsequent generations (4, 5). Proposed mechanisms include direct effects on germ cells in the first-generation (F1)[6] fetus by inheritance of persistent genetic or epigenetic modifications in a parent-specific manner or by abnormal pregnancy adaptations (6). However, it is unclear how early the second

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* To whom correspondence should be addressed. E-mail: m.wlodek@unimelb.edu.au.

[6] Abbreviations used: F0, generation naught, normal female or male animals; F1, first-generation; F2, second-generation; G2, second-stage blastocyst culture growth; GMOPS, 3-morpholinopropane-1-sulfonic acid-buffered first-stage blastocyst culture growth; HSA, human serum albumin; ICM, inner cell mass; mTOR, mammalian target of rapamycin; Pi3k, phosphoinositide 3 kinase; restricted, offspring born of low birth weight after bilateral uterine vessel ligation surgery; ^{1}H-NMR, hydrogen-1 nuclear magnetic resonance.

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generation may be affected, and whether alterations are detectable during early preimplantation development.

Our established rat model of uteroplacental insufficiency mimics intrauterine growth restriction similar to that observed in the Western world whereby oxygen and nutrient supply are reduced because of uteroplacental insufficiency (7, 8). Although both F1 male and female offspring have organ deficits, only F1 male rats develop hypertension and metabolic dysfunction in the absence of obesity (7, 9–12). We, and others, have also demonstrated that these deficits are not limited to F1 offspring and can be transmitted, by mothers born small, to the second generation (13–18). The second-generation (F2) transgenerational effects transmitted via the F1 paternal line after growth restriction are less well characterized.

Maternal and paternal, generation naught (F0), health and nutrition are known to affect F1 embryo development. Studies of F0 fathers exposed to high-fat diets have reported delays in F1 embryo growth, reduced blastocyst cell numbers, and increased rates of glycolysis (19). Paternal high-fat diet can result in F1 β-cell dysfunction, impaired glucose tolerance, and insulin resistance and can program F2 obesity, impaired insulin resistance, and metabolic defects (20–23). Of relevance, F0 maternal high-fat diet increased body size in 3 consecutive generations of female offspring, programmed through changes in paternally imprinted genes (24). Despite observations that paternal nutrient availability affects F1 preimplantation development, limited studies have characterized alterations in F2 preimplantation embryo growth and development.

The preimplantation period of embryo development represents a critical and sensitive 4-day developmental window in the rodent, resulting in an implantation-competent blastocyst. During this time, global gene expression is epigenetically programmed, with many genes affected by parental imprinting. These and other developmental processes can be affected by changes in tubal and uterine nutrients, particularly glucose and amino acids, which are actively used by the preimplantation blastocyst (25–28). It is reported that alterations to maternal diet in the rodent can substantially alter amino acid levels in maternal plasma and uterine fluid, altering blastocyst amino acid content, thereby affecting growth and development (29). Because the placenta is an important regulator of fetal growth after implantation and is largely driven by paternal gene expression, alterations caused by impaired trophectoderm development or function during the preimplantation period may affect fetal nutrition and growth, leading to the programming of disease risk in resultant progeny. In addition, cellular alterations inherent to the inner cell mass (ICM), influenced by either parental background, may persist from fertilization, further affecting fetal growth. The aims of this study were, therefore, to assess blastocyst structural characteristics and carbohydrate utilization, blastocyst outgrowth potential and amino acid metabolism, and the expression of genes related to growth in F2 blastocysts from F1 fathers whose fetal growth and weight at birth are impaired by surgically induced uteroplacental insufficiency.

Methods

Unless otherwise stated, chemicals were from Sigma.

Mating and animal generation. This study was approved by The University of Melbourne Animal Ethics Experimentation Committee (AEC 1112128) before all experimental procedures. A vaginal impedance reader (model MK-10B; Mukomachi Kikai) was used to determine the time of estrus for mating as described previously (7, 8). Female Wistar Kyoto rats (F0) were mated between 18 and 24 wk of age. On day 18 of gestation, rats underwent sham (control) or bilateral uterine vessel ligation (restricted) surgery (7, 8). Rats gave birth naturally at term (day 22) to F1 control (normal birth weight) and F1 growth-restricted offspring (born small), respectively, to study paternal line transmission. F1 males were removed from F0 mothers at weaning (day 35) and housed with another male until the time of mating. Body weights and dimensions were measured in F1 males at day 1, 7, 14, and 35 and at 2, 3, and 4 mo of age. Blood pressure of F1 male offspring was measured at 4 mo of age by using the tail cuff method as previously described (2).

F1 control and restricted males (only 1 male from each F1 litter), at 9–11 mo of age were naturally mated with normal virgin females (F0; 4 mo of age) in estrus (n = 10 per group). F0 normal virgin females were obtained from The University of Melbourne Animal Breeding Facility. Mating success was confirmed by the presence of a vaginal plug after mating, and the next morning was considered day 0.5 of pregnancy. All male and female body weights were measured before mating and at postmortem (day 4.5 of pregnancy) after they were killed by carbon dioxide inhalation (7, 8). Maternal plasma insulin concentrations were measured in duplicate with the use of a rat insulin radioimmunoassay kit (Millipore, Abacus ALS) (17). Maternal plasma glucose concentrations were measured in duplicate by using a scaled-down version of the enzymatic fluorometric analysis (17). For all experimental groups, 1 male per litter was studied. Three cohorts of males were generated for gene analyses (6 per group), blastocyst structural characteristics and carbohydrate utilization (6 per group), and outgrowth and amino acid utilization (6 per group).

RT^2 profiler PCR array analysis. Total RNA was extracted from 3 independent biological replicates of pooled blastocysts by using the Roche Total RNA Isolation Kit (Roche) (from 2 females per group, 10–20 blastocysts/dam to yield 3 independent biological replicates) according to the manufacturer's instructions. Briefly, samples were lysed and bound to a silica-based filter, treated with RNase-free DNase I (Roche), then washed with supplied kit buffer before elution in 50 μL of supplied elution buffer. RNA concentration was assessed with a NanoDrop absorbance spectrophotometer (ND1000; Thermo Scientific), and RNA integrity was evaluated with the Agilent 2100 Bioanalyzer by using a RNA 6000 Nano Assay Kit (Agilent Technologies). RNA was converted to cDNA by using the RT^2 First Strand cDNA synthesis kit (Qiagen) and amplified with a RT^2 PreAMP cDNA synthesis kit [Rat mammalian target of rapamycin (mTOR) Signaling PCR array and Rat Insulin Signaling Pathway PCR array; Qiagen] according to the manufacturer's instructions. Gene expression of mTOR and insulin signaling pathways were analyzed on respective RT^2 Profiler Arrays (Qiagen). Amplification was performed on an ABI Viia7 (Applied Biosystems Life Technologies) with resultant gene expression analyzed with the manufacturer's recommended Web-based PCR Array Data Analysis software (30).

Blastocyst flushing and culture. Day 4.5 blastocysts were retrieved from intact uteri of mated female rats by flushing with laboratory-made MOPS-buffered second-stage blastocyst culture growth (G2) medium (31) modified for the rat by increasing the osmolality to 310 mOsmol/kg with the use of sodium chloride, as described previously (n = 15–40 blastocysts per group from 6 females per group) (32). Blastocysts were then cultured in laboratory-made bicarbonate-buffered G2 medium (31) also with modified osmolality, supplemented with 5 mg/mL human serum albumin (HSA; Vitrolife AB) under paraffin oil (Ovoil; Vitrolife AB) and housed in a dual gas incubator (ThermoForma) at 37°C, in an atmosphere of 6% CO_2, 5% O_2, and 89% N_2 for 24 h (33). F2 blastocysts were scored for stage of development at flushing and again after the 24-h culture period, according to a numeric grade that was based on structural characteristics (grade 3, early blastocyst; grade 4, expanded blastocyst; grade 5, hatching blastocyst; grade 6, fully hatched blastocyst) (34). After 24-h culture, blastocysts were prepared for differential nuclear staining or outgrowth culture.

Differential nuclear staining. Trophectoderm and ICM cell numbers were assessed in F2 rat blastocysts by using a modification of a previously described method (n = 15–40 blastocysts per group from 6

females per group) (26, 35). Blastocysts were incubated in pronase [Sigma; 0.5% in MOPS-buffered first-stage blastocyst culture growth (GMOPS) media; 5 min at 37°C] to remove the zona pellucida, then incubated in picrylsulfonic acid (Sigma; 10 min at 37°C) and washed in GMOPS medium that contained 5 mg/mL HSA (Vitrolife AB). Embryos were then incubated in anti-dinitrophenol (Sigma; 10 min at 37°C), followed by washing in GMOPS media with HSA. Complement-mediated lysis was performed by a short incubation in guinea pig serum (IMVS; 10 min at 37°C) before transfer to a 0.1 mg/mL bisbenzimide solution (Hoecsht 33342; Sigma). Blastocysts were then mounted in glycerol, and nuclei were counted under ultraviolet light (filter) by using an inverted microscope (TS100-F; Nikon).

Blastocyst glucose consumption and lactate production quantification. Spent culture media samples were stored at −80°C after 24-h incubation with individual F2 blastocysts (n = 15–40 blastocysts per group from 6 females per group). Glucose and lactate concentrations were measured with enzyme-linked fluorescence assays (36, 37). For this, F2 blastocysts were first incubated in 4-μL drops of incubation medium, comprising bicarbonate-buffered G2 medium that was formulated with 0.5 mmol/L glucose as the sole substrate and no added lactate or pyruvate. After the 24-h incubation, glucose consumption and lactate production (expressed in mol/L) were quantitated per embryo by using a NanoDrop fluorospectrometer (ND3300; Thermo Scientific) and were normalized to the number of cells per embryo to account for differences in blastocyst cell numbers. Glycolytic activity (lactate:glucose ratio, expressed as percentage) was calculated per blastocyst, based on the assumption that 2 mol/L lactate is formed from 1 mol/L glucose (38).

Blastocyst outgrowth culture. F2 blastocyst outgrowths were performed as previously described (n = 30 blastocysts per group from 6 females per group) (39). Flat-bottomed 96-well plates (BD Biosciences) were coated with fibronectin (10 μg/mL; Sigma) and incubated with 4 mg/mL bovine serum albumin (Sigma) for 1 h. Wells were washed and filled with 150 μL of a modified G2 medium that was supplemented with 5 mg/mL HSA (Vitrolife AB) and equilibrated at 37°C under paraffin oil for 3 h. Hatched blastocysts were placed individually into coated wells and incubated for a period of 66 h. Blastocyst outgrowth was examined through the acquisition of images taken at 10× magnification at 4, 18, 23, 28, 42, 47, 52, and 66 h during culture, using an inverted microscope equipped with a heated stage at 37°C (Eclipse TS100-F; Nikon). The area of outgrowth was measured in each image by using NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon). All images were analyzed at matching magnification (10×). The average area of outgrowth was calculated for each treatment and replicated 3 times. At the completion of culture (66 h after transfer), 100 μL of outgrowth media was collected for hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) analysis.

Metabolomic analysis of culture media. Outgrowth media was analyzed for carbohydrate and amino acid composition by using ^{1}H-NMR spectroscopy (n = 30 blastocysts per group from 6 females per group). Chilled methanol (−20°C; 140 μL) was added to 70-μL aliquots of spent outgrowth medium from F2 control and restricted blastocyst cultures and was incubated on ice for 15 min (40, 41). Samples were then centrifuged at 5000*g* for 15 min. A 160-μL aliquot of supernatant fluid was collected and dried under speed vacuum for 12 h at 45°C. Samples were then resuspended in 540 μL of 200 mmol/L of trisodium phosphate in deuterium oxide (titrated to pH 7 with deuterium chloride; Sigma and Cambridge Isotope Laboratories Inc.). An additional 60 μL of 5 mmol/L 2,2-dimethyl-2-silapentane-5-sulfonic acid trisodium phosphate (Cambridge Isotope Laboratories Inc.) was added as a standard for determining concentrations of media components. The 600-μL final sample volume was added to 5-mm 507 grade glass NMR tubes (Wilmad LabGlass) before spectral acquisition.

Samples were analyzed on a 600-MHz Bruker Avance US^2 spectrometer (Bruker BioSpin Pty. Ltd.) equipped with a 5-mm triple resonance cryoprobe. Samples were locked to deuterium and were gradient shimmed. The 90° pulse width was calibrated, and receiver gain was optimized for each sample. Spectra were collected over 64-k data points and 256 scans. A 1-dimensional nuclear overhauser spectrometry pulse sequence with presaturation for water suppression was used (recycle delay, 90°; τ, 90°; τ_m, 90°; acquire free induction decay), with a recycle delay of 1.5 s, mixing time (τ_m) of 50 ms, and transmitter frequency offset of 2848 Hz. Spectra were Fourier transformed and phase corrected and then calibrated to the 2,2-dimethyl-2-silapentane-5-sulfonic acid signal at 0.00 ppm. A line-broadening factor of 0.5 Hz was applied to all spectra. Processed NMR spectra were imported into the Chenomx NMR Suite 6.1 (Chenomx Inc.), and media components were identified and quantified with the 600-MHz compound library. The NMR data acquisition and processing parameters were selected to maintain compatibility with the Chenomx metabolite library as described previously (41).

Statistical analysis. All developmental, gene expression, and microfluorescence data were analyzed by Mann-Whitney U test (SPSS Inc.). For all NMR data, metabolite concentrations were normalized to blastocyst outgrowth area by using Constant Sum normalization to correct for differences in metabolic flux due to blastocyst size (42, 43). A data matrix of quantified metabolites from ^{1}H-NMR spectral analysis of all samples was created. Scedasticity was corrected with $\log_{10}$ transformation. All transformed data were also analyzed with the Mann-Whitney U test (SPSS Inc.), with metabolite concentrations presented as production (positive values) or consumption (negative values) relative to the control media sample, in pixel^2/blastocyst per hour (pixel^2) ± SEM. All data are presented as means ± SEMs, and differences were considered significant when $P < 0.05$.

Results

Impact of uteroplacental insufficiency on F1 male physiology. Pregnant F0 females that underwent bilateral uterine vessel ligation gave rise to F1 growth-restricted male offspring that were born small and remained smaller at all subsequent time points assessed, compared with F1 control offspring ($P < 0.05$; **Table 1**). These males also had elevated blood pressure at 4 mo of age ($P < 0.05$; Table 1). Among the normal females used to generate F2 blastocysts, no differences were found in maternal age, weight, plasma glucose, plasma insulin, mating success, or the numbers of F2 blastocysts between control or restricted groups (Table 1).

Uteroplacental insufficiency in F1 males alters F2 blastocyst gene expression. qPCR analysis of mTOR and insulin signaling pathways revealed a reduction in the expression of several genes in F2 restricted blastocysts compared with control blastocysts at day 4.5 (9–74%; $P < 0.05$, **Figure 1**). These included reduced transcript levels of genes of the phosphoinositide 3 kinase (Pi3k) signaling family (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α gene, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit δ gene, and v-akt murine thymoma viral oncogene), cellular growth and proliferation genes (protein kinase, adenosine monophosphate-activated, β 2 non-catalytic subunit gene, tuberous sclerosis 1 gene, ras-related GTP binding A gene, ras-related GTP binding C gene, sterol regulatory element binding transcription factor 1 gene, serine/threonine kinase 11 gene, unc-51 like autophagy activating kinase 1 gene, mitogen-activated protein kinase kinase 1 gene, and ribosomal protein S6 kinase, 90 kDa, polypeptide 1 gene), and apoptosis genes (β-cell chronic lymphocytic leukemia/lymphoma 2-like 1 gene, growth factor receptor-bound protein 2-associated binding protein 1 gene, and tumor protein p53 gene). In addition, reduced expression was detected for miscellaneous genes, including serum/glucocorticoid regulated kinase 1 gene, involved in osmotic stress response;

TABLE 1 F1 control and restricted male rat variables, mating variables, and F2 control and restricted rat blastocyst measures[1]

Variable	Control	Restricted
F1 Males		
Body weight, g		
Day 1	4.4 ± 0.1	3.8 ± 0.1*
Day 7	11.4 ± 0.4	8.5 ± 0.5*
Day 14	24.5 ± 0.5	19.8 ± 1.0*
Day 35	88.2 ± 1.4	74.8 ± 2.1*
Month 2	245 ± 6	192 ± 7*
Month 3	321 ± 4	284 ± 5*
Month 4	357 ± 6	331 ± 6*
Blood pressure at 4 mo, mm Hg	136 ± 3	145 ± 3*
Mating age, wk	46 ± 5	46 ± 4
Mating weight, g	460 ± 6	417 ± 8*
F0 Females		
Age, wk	23 ± 1	25 ± 3
Weight, g	224 ± 3	219 ± 4
Plasma glucose, mmol/L	10.4 ± 2.4	11.6 ± 3.0
Plasma insulin, ng/mL	2.0 ± 0.4	1.7 ± 0.4
Mating success rate, %	100	100
Blastocysts collected, *n*/dam	8 ± 1	6 ± 1
F2 Blastocysts		
Grade at flush	5.2 ± 0.2	5.4 ± 0.2
Grade at incubation	5.9 ± 0.1	6.1 ± 0.0
Total nuclei, *n*	64 ± 4	64 ± 3
Trophectoderm, *n*	48 ± 4	47 ± 2
Inner cell mass, *n*	16 ± 1	17 ± 1
Glucose consumption, pmol/h		
Per embryo	36.7 ± 2.9	36.2 ± 1.5
Per cell	0.63 ± 0.05	0.57 ± 0.0
Lactate production, pmol/h		
Per embryo	38.5 ± 2.8	38.5 ±
Per cell	0.64 ± 0.04	0.6 0.04
Glycolytic rate, %		
Per embryo	56.6 ± 3.4	55.1 ± .6
Per cell	53.3 ± 3.9	56. ± 3.6

[1] Values are means ± SEMs, n = 10 or 15–40 blastocysts. *Significantly different from control, $P < 0.05$. Control, offspring born of normal birth weight after sham surgery; F0, generation naught, normal female or male animals; F1, first-generation; F2, second-generation; restricted, offspring born of low birth weight after bilateral uterine vessel ligation surgery.

adenylate cyclase-associated protein 1 gene, involved in cyclic AMP signaling; complement factor D gene, a trypsin-like protease; and coiled-coil domain containing 88B and myosin 1C genes, which code for proteins involved in microtubule-mediated organelle and vesicle transport ($P < 0.05$; Figure 1).

Absence of alterations in F2 blastocyst development and outgrowth potential. No differences were observable in cellular structural characteristics at the time of blastocyst retrieval or after 24 h in vitro culture between F2 control and restricted groups (Table 1). After 24-h incubation, no differences in total blastocyst cell numbers were observed (Table 1). Similarly, no alterations in the ICM:trophectoderm ratio of F2 restricted or control blastocysts were evident (data not shown). Furthermore, blastocyst outgrowth area over an additional 66-h period, beyond the first 24-h culture, did not differ between F2 control and restricted outgrowths (data not shown).

Metabolism is altered in F2 restricted blastocysts during trophoblast outgrowths. No differences were found in glucose consumption, lactate production, or glycolytic rates per blastocyst and, when normalized to blastocyst cell number, between F2 control and restricted blastocysts after 24-h culture (Table 1). However, after outgrowth, NMR analysis of culture media revealed that histidine, methionine, pyruvate, serine, and tyrosine consumption and aspartate and leucine production were significantly greater in the F2 restricted outgrowths than in control outgrowths ($P < 0.05$; **Table 2**).

Discussion

The findings from this study clearly indicate that male offspring born small, arising from uteroplacental insufficiency, program physiologic alterations that can manifest as modifications in gene expression levels and metabolic control of F2 blastocysts, even in the absence of overt cellular growth differences. These changes are likely to lead to subsequent adaptation of the implanting embryo and to influence ongoing development.

Although F1 growth-restricted male offspring have increased blood pressure as previously published (7, 12, 18), our model of growth restriction does not program obesity (17, 44). Previous studies have demonstrated that F0 paternal obesity adversely affects embryo development and function, which can lead to the development of F1 cardiovascular and metabolic dysfunction (19, 20, 24). The effects on F2 blastocysts described in this study are a direct result mediated by growth restriction and is not confounded by obesity, further strengthening the findings of this study.

Studies have suggested that alterations to embryo development may occur because of poor or altered maternal physiology. Maternal consumption of low-protein diets in rodents leads to blastocysts with lower numbers of cells in the ICM and trophectoderm due to slower proliferation (45). In rodent models of gestational diabetes, resultant embryos had alterations in glucose metabolism, which was related to decreases in F1 blastocyst cell number, developmental competence, and implantation potential (38, 46, 47). Because our F2 blastocysts were retrieved from normal females, they are not likely to have been exposed to compromised maternal physiology. This may explain the lack of acute growth-related differences observed in F2 blastocysts, indicated by similar structural characteristics, growth, and metabolic variables at the time of retrieval and after 24 h of culture.

Despite the absence of structural characteristics or growth differences, substantial changes in gene expression levels in F2 blastocysts derived from F1 growth-restricted fathers were observed. Importantly, many of the genes identified code for proteins associated with metabolic processes. For example, the mTOR-dependent signaling cascades are known to regulate trophectoderm motility (48, 49). This pathway is activated by amino acids and is responsible for cellular growth, proliferation, motility, protein synthesis, and transcription. Therefore, appropriate blastocyst nutrition, when there is an adequate supply of amino acids, is essential for normal blastocyst growth and development. The decreased expression of sterol regulatory element binding transcription factor 1 and serine/threonine kinase 11 genes may be because of deficiencies of specific amino acids in the blastocyst, alterations in transport of amino acids, or epigenetic changes to gene expression levels in the blastocyst.

The reduction in Pi3k genes, which encode kinases and binding genes (protein kinase, adenosine monophosphate-activated, β 2 non-catalytic subunit genes, v-akt, murine

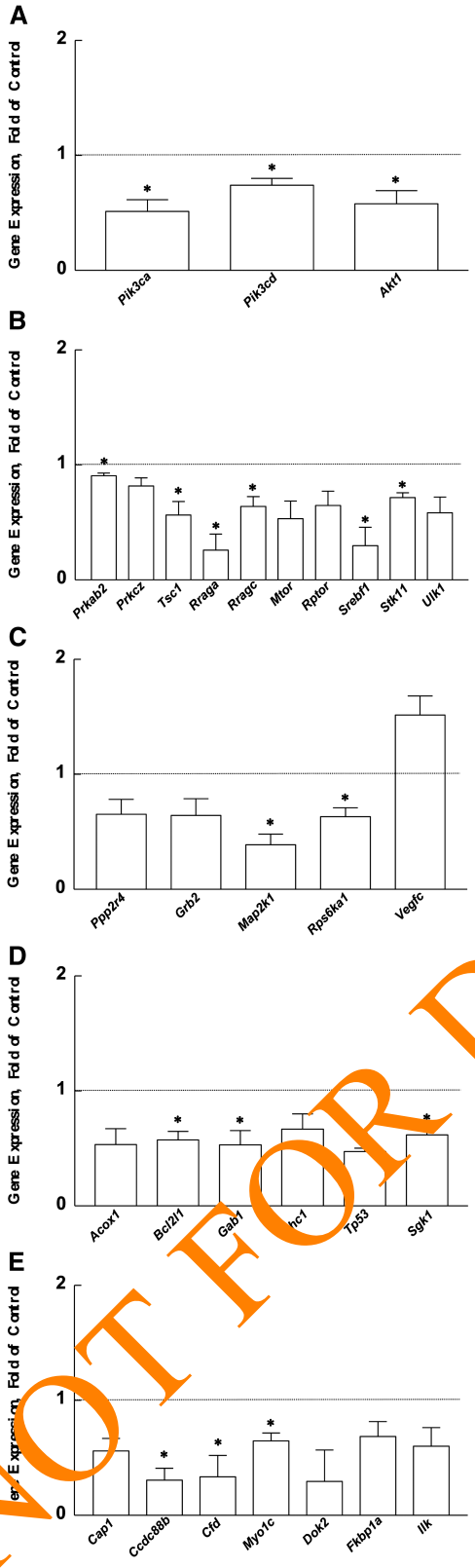

FIGURE 1 Gene expression in day 4.5 F2 blastocysts derived from F1 control and restricted male rats. (A) Pi3k; (B) Pkb/Akt; (C) growth and motility; (D) B-oxidation, apoptosis, and osmotic stress; and (E) other relative gene expression to control amount (1, dotted line). All data are presented as means ± SEMs, $n = 3$ independent biological replicates from 2 females per group (10–20 blastocysts/dam). *Significantly different from control, $P < 0.05$. *Acox1*, acyl-CoA oxidase 1, palmitoyl gene; *Akt1*, v-akt murine thymoma viral oncogene; *Bcl2l1*, β-cell chronic lymphocytic leukemia/lymphoma 2-like 1 gene; *Cap1*, adenylate cyclase-associated protein 1 gene; *Ccdc88b*, coiled-coil domain containing 88B gene; *Cfd*, complement factor D gene; control, offspring born of normal birth weight after sham surgery; *Dok2*, docking protein 2 gene; *Fkbp1a*, FK506 binding protein 1a gene; F1, first-generation; F2, second-generation; *Gab1*, growth factor receptor-bound protein 2-associated binding protein 1 gene; *Grb2*, growth factory receptor-bound protein 2 gene; *Ilk*, integrin-linked kinase gene; *Map2k1*, mitogen-activated protein kinase kinase 1 gene; *Mtor*, mechanistic target of rapamycin gene; *Myo1c*, myosin 1C gene; *Pik3ca*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α gene; *Pik3cd*; phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit δ gene; Pi3k, phosphoinositide 3 kinase; Pkb/Akt, serine/threonine protein kinase B; *Ppp2r4*, protein phosphatase 2A activator, regulatory subunit 4 gene; *Prkab2*, protein kinase, adenosine monophosphate-activated, β 2 non-catalytic subunit gene; *Prkcz*, protein kinase C ζ gene; restricted, offspring born of low birth weight after bilateral uterine vessel ligation surgery; *Rps6ka1*, ribosomal protein S6 kinase, 90 kDa, polypeptide 1 gene; *Rptor*, regulatory-associated protein of MTOR, complex 1 gene; *Rraga*, ras-related GTP binding A gene; *Rragc*, ras-related GTP binding C gene; *Sgk1*, serum/glucocorticoid regulated kinase 1 gene; *Shc1*, Src homology 2 domain-containing transforming protein 1 gene; *Srebf1*, sterol regulatory element binding transcription factor 1 gene; *Stk11*, serine/threonine kinase 11 gene; *Tp53*, tumor protein p53 gene; *Tsc1*, tuberous sclerosis 1 gene; *Ulk1*, unc-51 like autophagy activating kinase 1 gene; *Vegfc*, vascular endothelial growth factor C gene.

thymoma viral oncogene, and tuberous sclerosis 1 gene) may affect Pi3k signaling, thereby affecting growth pathways. Upstream, Pi3k pathways are also known to be activated by receptor-mediated binding of growth factors such as insulin growth factors and can affect amino acid regulation. Hence, downregulation of this pathway may reflect a response to deficiencies of specific growth and differentiation regulation by the blastocyst, although development may be compensated for by upregulation of other signaling pathways, or co-activation of receptors by other ligands. Downstream, Pi3k signaling affects serine/threonine protein kinase B (50–52), whose reduced activity is related to defects in placental development and impaired fetal growth (53). Conceivably, altered Pi3k regulation may result in blastocysts that develop in a timely manner but have suboptimal implantation and trophectoderm differentiation capacity.

Reduced expression of genes involved in apoptosis and osmotic stress in F2 restricted blastocysts, may be related to the ability of the trophectoderm to maintain blastocoel integrity and therefore ICM homeostasis. Apoptosis influences trophoblast differentiation and remodeling of the placenta (54). Tumor protein p53 and β-cell chronic lymphocytic leukemia/lymphoma 2-like 1 genes are also implicated in mitochondrial function. Inherent impairments in signaling may also be related to changes in adenylate cyclase-associated protein 1, coiled-coil domain containing 88B, and myosin 1C gene expression in F2 restricted blastocysts. Because these transport processes are also ATP dependent, their downregulation may indicate transient changes in ATP availability, affecting blastocyst formation. However, because blastocyst growth was unaffected by paternal growth restriction, this suggests that the embryo has adapted its metabolism and has perhaps developed other ways to generate sufficient ATP to support blastocyst formation, or effects may affect later stage embryo development.

Consistent with the observed changes in gene expression, F2 restricted outgrowths derived from males born small displayed changes in nutrient metabolic flux. The increased consumption of specific amino acids, notably histidine, methionine, serine,

TABLE 2 Metabolite utilization by day 8 F2 blastocysts derived from F1 control and restricted male rats[1]

Metabolite, pM/blastocyst per hour (pixels^2)	Control	Restricted
Glucose	−1.48 ± 2.09	−2.39 ± 5.06
Lactate	−19 ± 20	−30 ± 19
Pyruvate	0.22 ± 0.09	−0.07 ± 0.16*
Essential amino acids		
Arginine	1.78 ± 0.75	−5.48 ± 3.76
Histidine	−0.04 ± 0.14	−0.42 ± 0.46*
Isoleucine	−0.58 ± 0.77	−2.35 ± 1.29
Leucine	0.15 ± 0.19	0.25 ± 0.47*
Lysine	−1.19 ± 1.28	−2.74 ± 1.52
Methionine	0.003 ± 0.27	−1.26 ± 0.61*
Phenylalanine	−0.34 ± 0.32	−1.98 ± 1.03
Threonine	−2.07 ± 2.46	−8.68 ± 4.73
Valine	−0.91 ± 0.95	−2.55 ± 1.35
Nonessential amino acids		
Alanine	−0.20 ± 0.43	−1.84 ± 1.13
Asparagine	1.21 ± 1.05	2.51 ± 1.09
Aspartate	0.56 ± 0.41	0.75 ± 0.28*
Cysteine	1.39 ± 1.11	2.32 ± 1.54
Glutamate	0.02 ± 0.27	−2.21 ± 1.48
Glycine	−5.53 ± 4.94	−10.9 ± 6.55
Proline	1.88 ± 1.34	−1.89 ± 1.46
Serine	0.09 ± 0.22	−3.86 ± 1.76*
Tyrosine	−0.48 ± 0.37	−0.88 ± 0.47*

[1] Values are means ± SEMs; n = 30 blastocysts per group. *Significantly different from control, $P < 0.05$. Metabolite concentrations are presented as production (positive values) or consumption (negative values) relative to the control media sample. Control, offspring born of normal birth weight after sham surgery; F1, first-generation; F2, second-generation; restricted, offspring born of low birth weight after bilateral uterine vessel ligation surgery.

and tyrosine, is similar to mouse blastocysts grown under suboptimal culture conditions (55). These changes in overall growth amino acid utilization occurred at a time that equates to early development after implantation, when the trophectoderm plays a major role in blastocyst attachment, implantation, and subsequent placental formation. Serine was reported to be localized in the cytoplasm of placental cells and amounts increase during pregnancy (56), functioning to modulate intracellular growth factors and extracellular matrix proteins that regulate placental formation and function (56). As an adaptive mechanism, placental nutrient availability and utilization regulates amino acid and glucose transport activity (57, 58). Human intrauterine growth restriction often involves alterations in the activity of placental amino acid transport systems, such as system A, which is linked to alterations in mTOR protein amounts (59, 60). Signaling of mammalian target of rapamycin complex 1 is reported to be mediated through leucine and arginine in the blastocyst to control trophoblast motility (48, 49). Babies small for gestational age have reduced plasma concentrations of valine, leucine, and isoleucine (61). Studies of heat stress and intrauterine growth restriction commonly report reduced transplacental leucine flux and an increase in protein catabolism (62, 63). F2 restricted blastocysts, from growth-restricted males, displayed increased production amounts of aspartate and leucine in the present study, perhaps as a precursor to altered establishment of placental amino acid transport systems. In the case of aspartate, one of the most highly consumed amino acids in the blastocyst, alterations in its uptake may interfere with metabolic pathways necessary to maintain appropriate fetal development, as reported in the mouse (64). Conversion of histidine to histamine indirectly contributes to blastocyst-uterine signaling at implantation and is also implicated in blastocyst growth (65)

Finally, the impact of changes in amino acids has direct relevance to the regulation of epigenetic pathways, whereby alterations in serine and methionine may directly alter epigenetic marks of the developing embryo. Carbon atoms from amino acids such as serine and methionine are required for the folate cycle, which provides an intracellular mechanism for methyl-group transport and thereby methylation (66). In addition to the epigenetic reprogramming that may occur in the F2 embryo, epigenetic programming of (F1) sperm is also susceptible to environmental conditions. Studies of prenatal undernutrition in mice have highlighted modifications in male germ line epigenetic reprogramming, which permanently alters DNA methylation in F1 sperm (67). Alterations in nutritional status alter the transcriptional profile of the testis and mature F1 sperm content (22). This is in accordance with other paternal high-fat studies in which testicular gene expression was altered in F1 offspring (23, 68). The aim of the present study was to determine the effects in F2 blastocysts; however, ongoing studies are aimed at assessing F1 gonadal development and reproductive function in F1 growth-restricted male offspring. Resultant sperm from F1 offspring can then influence F2 embryonic growth and development after fertilization. Because we have characterized alterations in the F2 embryo before implantation, this in and of itself is suggestive of changes to placental development and function with arising alterations in sperm development, although this requires further investigation.

In conclusion, F1 growth-restricted fathers program next-generation disease risks via alterations in the mTOR pathway signaling and altered amino acid metabolism in F2 preimplantation blastocysts. These modifications, which may be epigenetic in nature, could have broad implications for the developmental origins of adult health and disease hypothesis.

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JSM, DKG, and MEW designed the research; JSM conducted the research; JSM, GAT, AJH, JRS, and NJH analyzed the data; JSM wrote the paper. JSM, GAT, AJH, JRS, NJH, DKG, and MEW take responsibility of data interpretation and presentation. All authors read and approved the final manuscript.

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