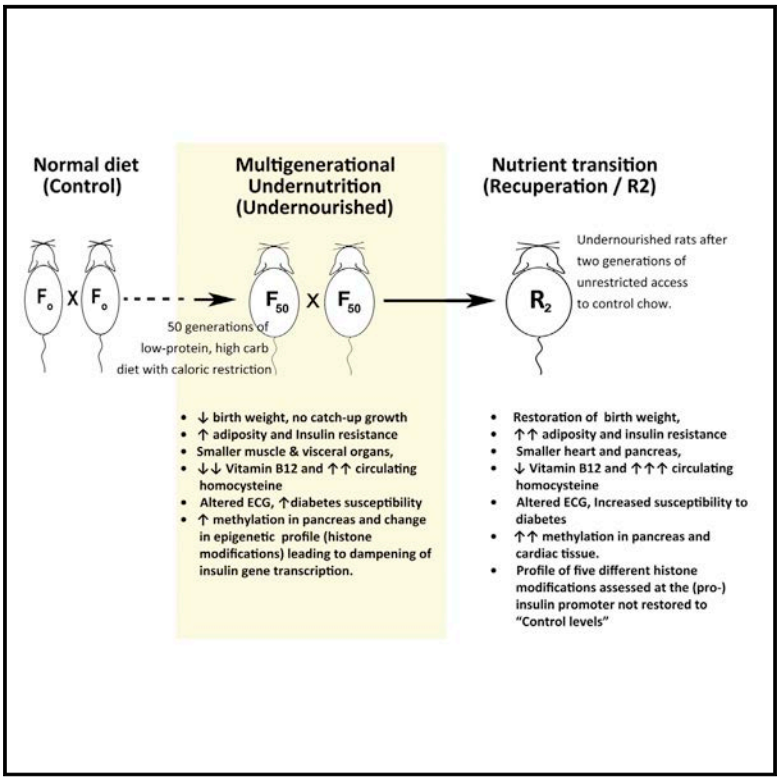
Short Article

Cell Metabolism

Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation

Graphical Abstract



Highlights

- Undernourished rats are protein / calorie-restricted for 50 generations
- Recuperation rats are generated by feeding normal chow for two more generations
- Undernourished and Recuperation rats show multiple markers of metabolic disease
- Metabolic / epigenetic alterations are not reversed following nutrient recuperation

Authors

Anandwardhan A. Hardikar, Sarang N. Satoor, Mahesh S. Karandikar, ..., Anthony C. Keech, Alicia J. Jenkins, Chittaranjan S. Yajnik

Correspondence

anand.hardikar@ctc.usyd.edu.au

In Brief

In a rat model of undernutrition over 50 generations, closely mimicking human populations in developing countries, Hardikar et al. show that undernourished rats display metabolic abnormalities associated with epigenetic changes, which are not reversed following unrestricted access to normal chow in two subsequent generations.

Hardikar et al., 2015, Cell Metabolism *22*, 312–319
August 4, 2015
http://dx.doi.org/10.1016/j.cmet.2015.06.008

CellPress

CellPress

Cell Metabolism
Short Article

Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation

Anandwardhan A. Hardikar,[1,11,*] Sarang N. Satoor,[1,2,11] Mahesh S. Karandikar,[3,4,11] Mugdha V. Joglekar,[1,11] Amrutesh S. Puranik,[2,12] Wilson Wong,[1] Sandeep Kumar,[2] Amita Limaye,[2,5] Dattatray S. Bhat,[6] Andrzej S. Januszewski,[1] Malati R. Umrani,[2] Amaresh K. Ranjan,[7] Kishori Apte,[8] Pranav Yajnik,[9] Ramesh R. Bhonde,[2,10] Sanjeev Galande,[5] Anthony C. Keech,[1] Alicia J. Jenkins,[1] and Chittaranjan S. Yajnik[6]

[1]NHMRC Clinical Trials Centre, University of Sydney, Sydney, NSW 2050, Australia
[2]National Center for Cell Science, Ganeshkhind Road, Pune 411007, India
[3]Department of Physiology, DY Patil Medical College, DPU, Pimpri, Pune 411018, India
[4]Department of Physiology, BJ Medical College, Pune 411011, India
[5]Indian Institute of Science Education and Research (IISER), Dr Homi Bhabha Road, Pashan, Pune 411008, India
[6]Diabetes Unit, KEM Hospital, Rasta Peth, Pune 411011, India
[7]Cardiovascular Institute, Mount Sinai School of Medicine, New York, NY 10029, USA
[8]National Toxicology Center, 36/1/1 MN199, Vadgaon Khurd, Singhgad Road, Pune 411041, India
[9]Department of Biostatistics, University of Michigan, Ann Arbor, Michigan 48109, USA
[10]Manipal Institute of Regenerative Medicine, Manipal University, Bangalore, India
[11]Co-first author
[12]Present address: Robert & Arlene Kogod Center on Aging, Mayo Clinic, Rochester, MN 55905, USA
*Correspondence: anand.hardikar@ctc.usyd.edu.au
http://dx.doi.org/10.1016/j.cmet.2015.06.008

SUMMARY

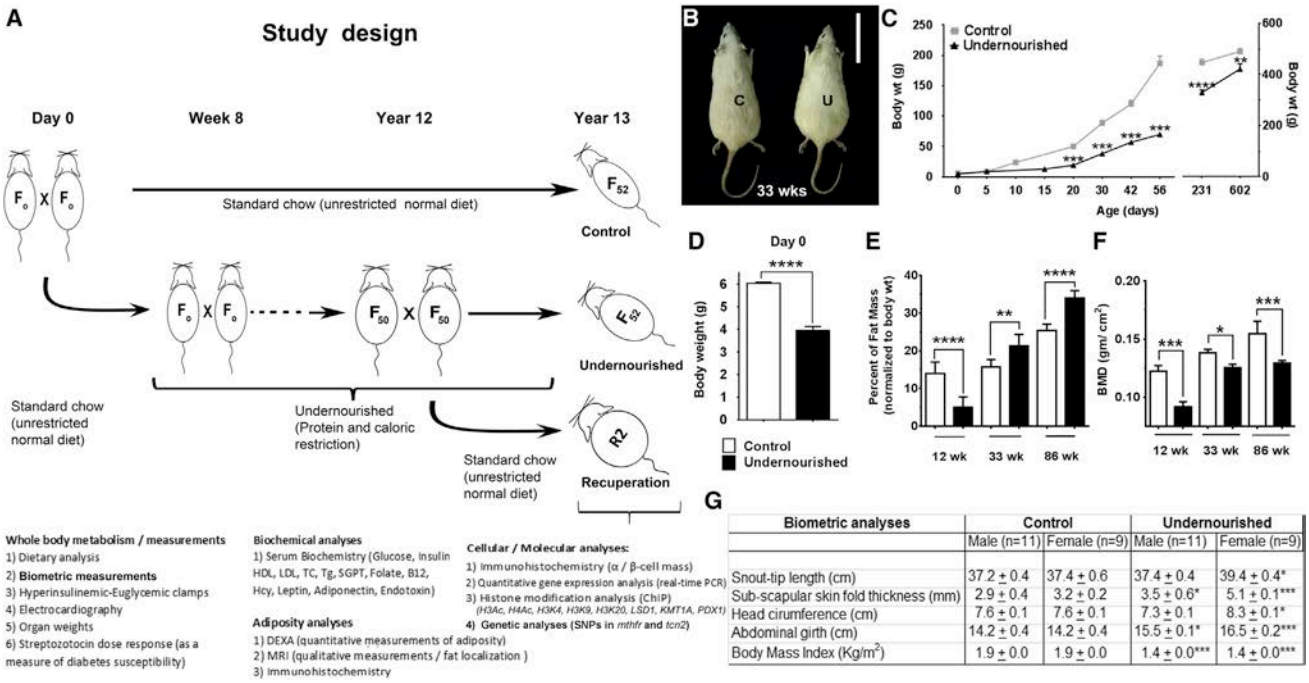
People in developing countries have faced multigenerational undernutrition and are currently undergoing major lifestyle changes, contributing to an epidemic of metabolic diseases, though the underlying mechanisms remain unclear. Using a Wistar rat model of undernutrition over 50 generations, we show that Undernourished rats exhibit low birth-weight, high visceral adiposity (DXA/MRI), and insulin resistance (hyperinsulinemic-euglycemic clamps), compared to age-/gender-matched control rats. Undernourished rats also have higher circulating insulin, homocysteine, endotoxin and leptin levels, lower adiponectin, vitamin B12 and folate levels, and an 8-fold increased susceptibility to Streptozotocin-induced diabetes compared to control rats. Importantly, these metabolic abnormalities are not reversed after two generations of unrestricted access to commercial chow (nutrient recuperation). Altered epigenetic signatures in insulin-2 gene promoter region of Undernourished rats are not reversed by nutrient recuperation, and may contribute to the persistent detrimental metabolic profiles in similar multigenerational undernourished human populations.

INTRODUCTION

The burden of type 2 diabetes mellitus (T2D) is increasing worldwide, particularly in developing countries, where >70% of the global burden of T2D is predicted to exist by 2030 (Echouffo-Tcheugui and Dagogo-Jack, 2012). Although reasons for the increasing rates of T2D in developing countries are not fully elucidated, important factors include lifestyle changes involving rural-to-urban migration (“urbanization”), intra-uterine undernutrition, and fetal programming.

During the past two decades, increasing evidence arising from multiple clinical studies conducted by the research teams of Yajnik and Barker support an important role of early life undernutrition, and specifically disturbances of one-carbon metabolism, in the heightened susceptibility of (Asian) Indians to T2D at a younger age, and in the absence of generalized obesity (Yajnik et al., 1995, 2003, 2014; Yajnik and Deshmukh, 2012). These studies have highlighted body composition and nutritional-metabolic peculiarities of multigenerationally undernourished Indians: a thin-fat (low lean mass, high fat mass) phenotype compared to Europeans, with predominant visceral deposition of fat. This body composition is strongly associated with insulin resistance and related metabolic-endocrine abnormalities. Importantly, this “thin-fat” phenotype was present at birth and, therefore, programmed during intrauterine life, possibly through epigenetic mechanisms over multiple generations. Maternal intergenerational undernutrition, evident in stunting, low BMI, and a disturbance of dietary methyl donors (low protein and vitamin B_{12} and high folate status, related to vegetarian diets) appear contributory to the increased risk of diabetes and CVD in Indians (Yajnik, 2004; Yajnik and Deshmukh, 2012; Yajnik et al., 2003, 2008).

It is now well appreciated that the intra-uterine environment can induce heritable alterations that may be retained over generations (Aiken and Ozanne, 2014; Goodspeed et al., 2015; Ng et al., 2010). In non-human primates, a maternal high-fat diet supplemented with calorically dense treats leading to obesity



G

Biometric analyses	Control		Undernourished	
	Male (n=11)	Female (n=9)	Male (n=11)	Female (n=9)
Snout-tip length (cm)	37.2 ± 0.4	37.4 ± 0.6	37.4 ± 0.4	39.4 ± 0.4*
Sub-scapular skin fold thickness (mm)	2.9 ± 0.4	3.2 ± 0.2	3.5 ± 0.6*	5.1 ± 0.1***
Head circumference (cm)	7.6 ± 0.1	7.6 ± 0.1	7.3 ± 0.1	8.3 ± 0.1*
Abdominal girth (cm)	14.2 ± 0.4	14.2 ± 0.4	15.5 ± 0.1*	16.5 ± 0.2***
Body Mass Index (Kg/m^2)	1.9 ± 0.0	1.9 ± 0.0	1.4 ± 0.0***	1.4 ± 0.0***

Figure 1. Generation of a Multigenerational Undernourished Rat

(A) Study design illustrating the period of undernutrition and nutrient transition (Recuperation).

(B–F) (B) Control (C) and Undernourished (U) rats; bar, 10 cm, (C) growth curves of Control and multigenerational Undernourished rats showing low birth-weight (D) and no catch-up growth (C), (E) body fat and (F) bone mineral density measured using DXA at 12, 33, or 86 weeks.

(G) biometric measurements in Control and Undernourished rats; n ≥ 8, >4 litters, data presented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.01, and ****p < 0.0001; all comparisons against Controls.

has been shown to epigenetically alter chromatin structure in their progeny via SIRT1-mediated covalent modifications of histones (Aagaard-Tillery et al., 2008; Suter et al., 2012). Increased adiposity and insulin resistance have also been reported in high-fat diet-fed rodent models. Intra-uterine programming may involve epigenetic changes, which can be passed over generations, and may promote the development of adiposity and T2D.

In a preliminary study of naturally occurring food-deprived (for 12 years) Wistar rats, we identified differences in body composition and defects in glucose-insulin metabolism. We therefore decided to study the above phenotype and underlying mechanisms by replicating the diets in this prospective hypothesis-driven study. We present herein the first direct evidence that Wistar rats that are protein calorically undernourished over multiple (50) generations show increased adiposity, insulin resistance, and susceptibility to Streptozotocin (STZ)-induced diabetes. We further demonstrate that this adverse metabolic state is associated with altered histone modification profiles, which cannot be reversed by two generations of nutrient recuperation.

RESULTS AND DISCUSSION

A Multigenerational Rat Model of Undernutrition

Wistar rats were maintained for 50 generations (Figure 1A; Figure S1A) with unrestricted access to standard commercial chow (“Control”) or restricted to 50% of ad libitum mass of a chow containing 2.2-fold less protein, 1.3-fold more carbohydrates, 2.1-fold less fat, and 2.4-fold less fiber (Tables S1A, and S1B) with low vitamin supplementation (Table S1C), as compared to Control chow. The Undernourished (U) rats were lighter than Control (C) rats (Figures 1B and 1C), had low birth weight (Figure 1D), and did not show any catchup growth (Figure 1C). Dual-energy X-ray absorptiometry (DXA) measurements demonstrated that Undernourished rats had less body fat (normalized to body weight) than Control rats at 12 weeks of age but increased and exceeded control levels significantly at 33 and 86 weeks of age (Figure 1E). Their bone mineral density (BMD) was lower than Control rats at all times (Figure 1F). Biometric assessment demonstrated increase in skin-fold thickness, abdominal girth, and BMI following multigenerational undernutrition (Figure 1G). Thus, undernutrition over 50 generations led to a phenotype that was lighter at birth, failed to show catchup growth, and demonstrated increasing adiposity later in life.

Attempting to Correct Metabolic Effects of Multigenerational Undernutrition

After 50 generations of undernutrition, Undernourished rats were provided with unrestricted access to a standard (Control) chow diet from day 0 of pregnancy, and their progeny were studied at second generation of recuperation (R2 rats). These rats (Figure 2A) showed restoration of birth weight (Figure 2B, inset; Figures S1B and S1C) to Control values but grew significantly heavier than age-/gender-matched Control rats (Figures 2A

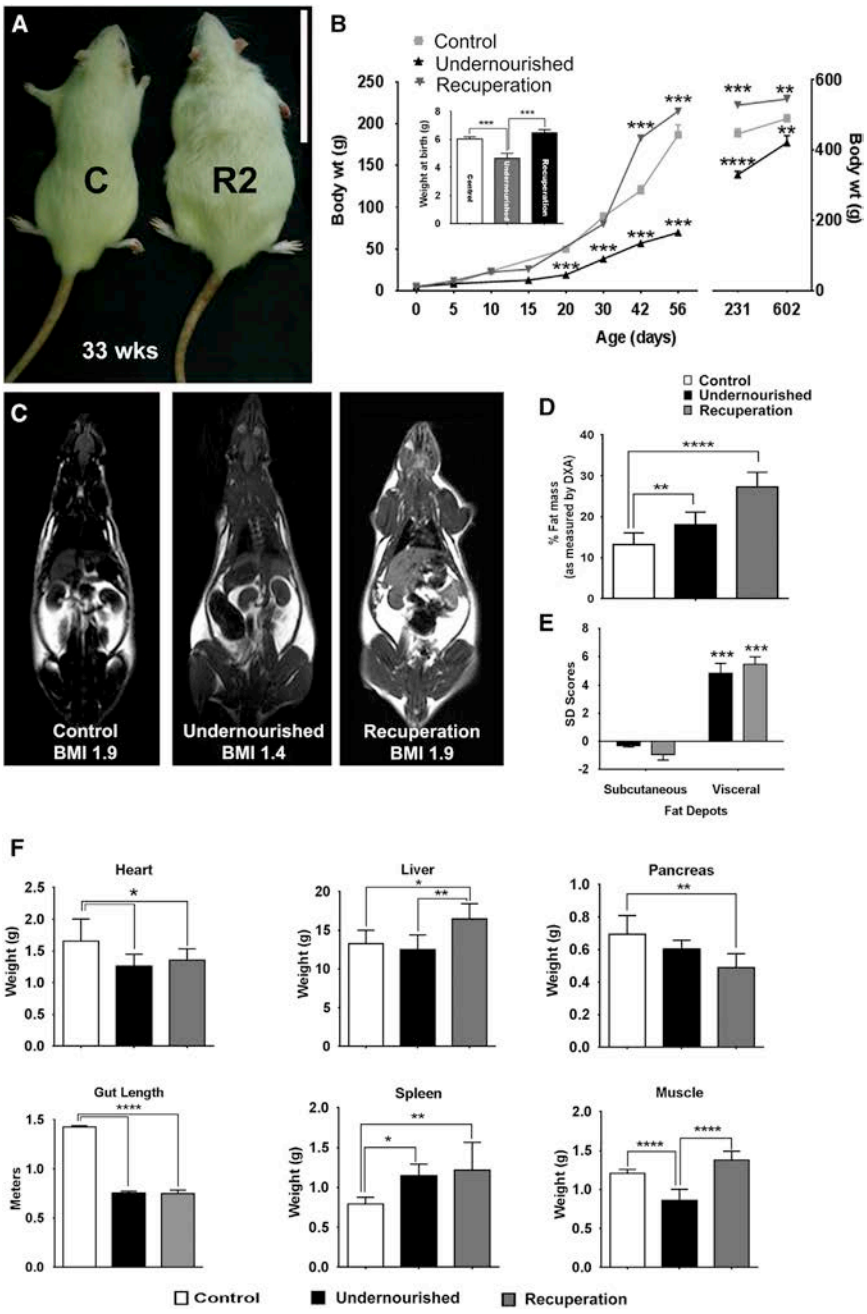


Figure 2. Nutrient Recuperation in a Multigenerational Undernourished Rat

(A and B) (A) Control (C) and second generation Recuperation rat (R2) (B) R2 rats show improvement in birth weight (versus Undernourished; inset in B), but significantly higher body weight post-weaning.

(C) MRI for the 33 week Control, Undernourished, and R2 rats; white areas represent fat distribution.

(D and E) (D) Fat mass measured by DXA showed significant increased adiposity in 33-week-old Undernourished and R2 rats, which is (E) mainly visceral (presented as SD scores, relative to Control).

(F) Organ weights of 33-week-old rats (normalized to body weight). Data presented as mean ± SEM, $n \geq 6$ (4 litters) (B–F), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; all comparisons against Controls.

and 2B) after the post-weaning period (Figure 2B; Figures S1D and S1E). Recuperation (R2) rats had higher abdominal girth on day 4 (Figure S1F) and the highest fat mass of the three groups (Control, Undernourished, and Recuperation) at 12 and 33 weeks of age (Figure S1G). MRI identified increased fat deposition in visceral organs of R2 rats, especially the liver (Figure 2C; Movie S1). DXA measurements confirmed significantly higher body fat in Undernourished and Recuperation rats than Controls (Figure 2D), most of which was due to visceral adiposity (Figure 2E). Multigenerational undernutrition thus appears to support mechanisms favoring accumulation of body fat (Stewart et al., 1980; Wells, 2006) as an adaptive mechanism (the so-called "Thrifty phenotype" hypothesis). However, the adaptive mechanisms of the Undernourished rats were not suited to the changing (Recuperation) environment of unrestricted access to Control chow. Recuperation rats showed restoration of birth weight but heavier body mass, a lighter heart and pancreas, and a heavier liver and spleen compared with Control rats (Figure 2F). Increased hepatic weight was mostly a result of fat accumulation (Figures S2A and S2B), which may contribute to increased splenic weight (Francque et al., 2011; Murray et al., 1986). Brain weight (data not shown) was similar to that in Control rats (both genders). Undernourished rats had smaller muscle mass. Interestingly, the gut length was shorter in Undernourished rats and remained shorter in R2 rats. Protein-deprivation in rats has been shown to lead to shorter intestines (Kasai et al., 2012). Similar changes induced over multiple generations of undernutrition in our study appear to introduce heritable alterations that were not reversed after two generations of "normal" diet. No gender differences were observed (Figures S1B–S1E, S2B, S2G–S2J, S4A, and S4B).

Unrestricted Access to a Control Diet in Multigenerational Undernourished Rats Promotes Adverse Metabolic Health in Later Life

We hypothesized that Undernourished rats provided with unrestricted access to Control commercial chow would present with metabolic profiles that are comparable to Control rats. Undernourished rats (relative to Controls) demonstrated similar circulating concentrations of serum endotoxin (Table S1D) but higher concentrations of circulating glucose ($p \leq 0.01$), insulin

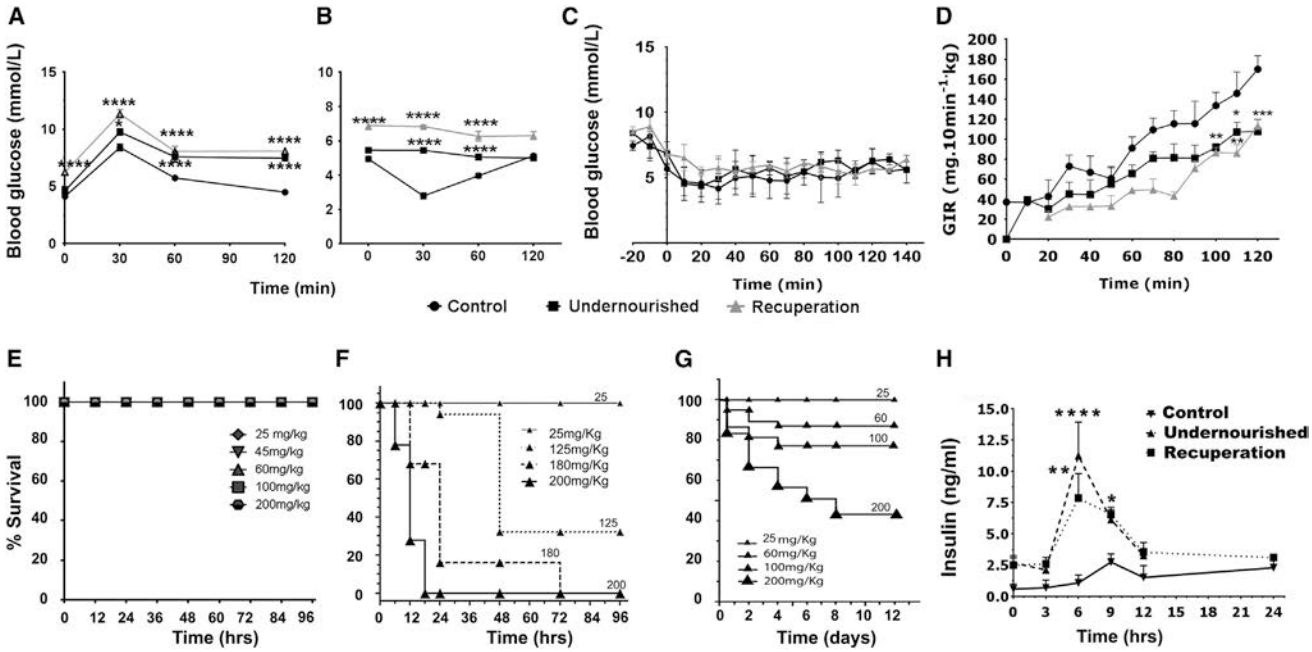


Figure 3. Insulin Sensitivity and Susceptibility to STZ-Induced Diabetes

(A–D) (A) Glucose tolerance test, (B) insulin tolerance test, (C) glucose was clamped during hyperinsulinemic-euglycemic clamp, and (D) glucose infusion rate (GIR) was measured during the clamp.

(E–G) Survival curves for Streptozotocin (STZ) dose response in (E) Control, (F) Undernourished, and (G) R2 rats.

(H) Circulating insulin after STZ injection (200 mg/kg). Data presented as mean ± SD, n ≥ 6 (4–12 litters). (A, B, and E–H) 14- to 20-week-old rats; (C and D) 33-week-old rats; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; all comparisons against Controls.

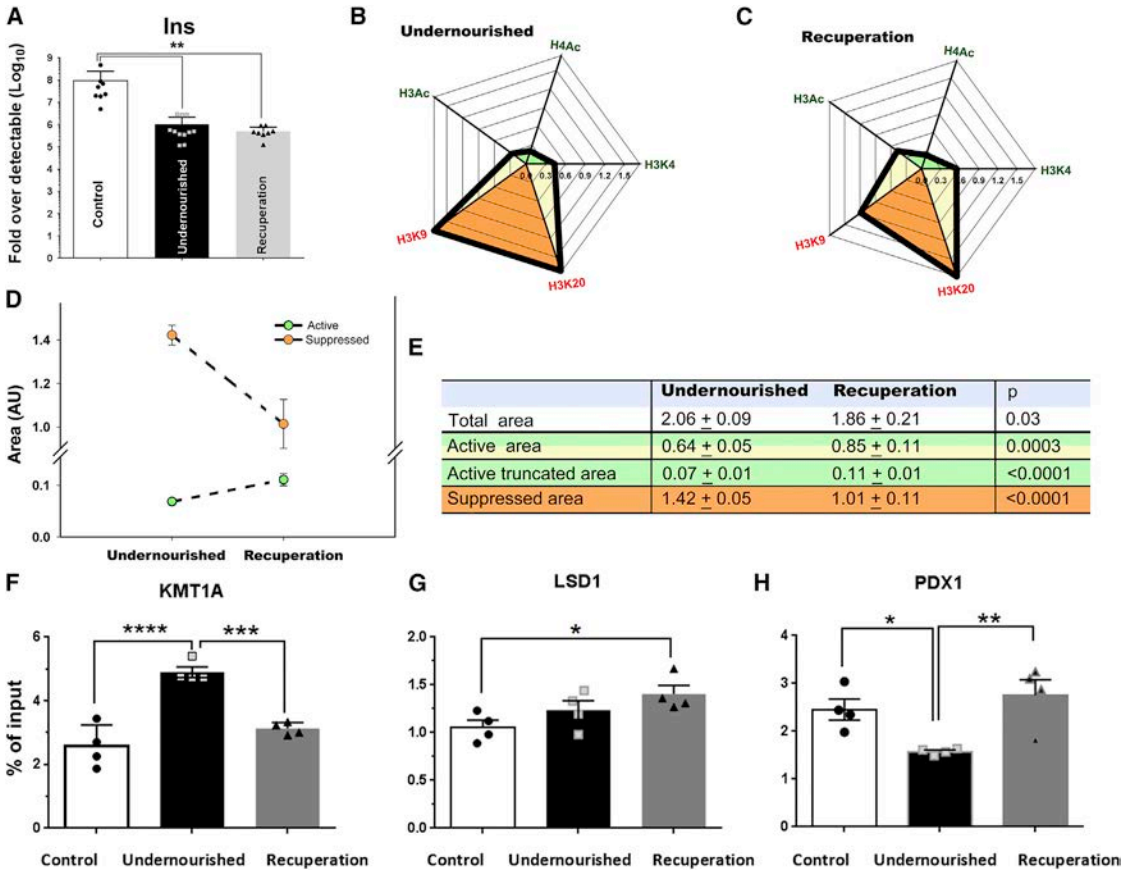
($p \leq 0.001$), leptin ($p \leq 0.05$), serum glutamic pyruvic transaminase (SGPT; $p \leq 0.001$), total homocysteine (tHcy; $p \leq 0.0001$) triglycerides (TG; $p \leq 0.0001$), and reduced concentrations of high-density lipoprotein-(HDL) cholesterol, folate, and vitamin B_{12} ($p \leq 0.0001$; all). In comparison with Control rats, Recuperation rats demonstrated elevated circulating insulin, glucose ($p \leq 0.0001$; both), leptin ($p \leq 0.001$), endotoxin ($p \leq 0.0001$), TG ($p < 0.0001$), total cholesterol ($p \leq 0.0001$), VLDL-cholesterol ($p \leq 0.0001$), LDL-cholesterol ($p \leq 0.001$), SGPT ($p \leq 0.01$), and tHcy ($p \leq 0.0001$), but similar levels of HDL-cholesterol (Table S1D).

Higher levels of SGPT in Undernourished and R2 rats compared to Control rats was consistent with liver damage due to fat deposition. We also observed that low levels of circulating vitamin B_{12} and folate in Undernourished rats were partially corrected in R2 rats. Macro-nutrient sufficiency thus seems to offer a considerable correction for vitamin B_{12} and folate deficiency as seen in the R2 rats, yet their levels remained significantly lower than Controls. Serum total homocysteine was elevated in Undernourished rats (versus Control) and did not reverse in R2 rats. Recuperation rats were visibly obese (Figure 2A) and showed sedentary habits as compared to Control rats (Movies S2 and S3), despite having similar total energy intake (Table S1B). Higher circulating leptin and lower adiponectin (Table S1D) levels in Undernourished and R2 rats reflected increased adiposity in these rats (Figures 1E and 2D; Figures S1F and S1G). Serum endotoxin concentrations were significantly higher in the R2 rats (Table S1D), as seen in human studies of obese, IGT, and T2D subjects (Harte et al., 2012). Similar to findings in mouse studies (Smith et al., 1966), elevations in serum endotoxin levels, along with hepatic fat (discussed above), may contribute to heavier spleens (Figure 2F) in Undernourished and R2 rats.

Fasting hyperinsulinemia was a prominent feature of Undernourished and R2 rats although islet insulin content was ~3-fold lower in Undernourished, but not R2, rats (Table S1D). We observed significant increases in numbers of insulin-containing cells in Undernourished and R2 rats with relatively fewer glucagon-containing cells (Figures S2C–S2E), though no significant increases in beta cell mass (Figure S2F) were observed.

Altered Metabolic Health following Multigenerational Undernutrition Is Not Restored through Macronutrient Supplementation

Following assessment of impaired glucose tolerance (Figures 3A and 3B; Figures S2G and S2H), hyperinsulinemic-euglycemic clamp studies were performed on Control, Undernourished, and R2 rats (Figures 3C and 3D; Figures S2I–S2J) to confirm insulin resistance. Significantly lower glucose infusion rates supported maintenance of clamped glucose concentrations in the Undernourished and R2 rats, confirming that the insulin resistance observed in the Undernourished rats was not restored following two generations of Control diet restoration. To understand whether undernutrition over generations altered the susceptibility to diabetes, we carried out a streptozotocin (STZ) dose response (see Experimental Procedures, Figure S3A). STZ, a pancreatic beta cell toxin, is routinely used to induce diabetes in Wistar rats. Undernourished rats died



	Undernourished	Recuperation	p
Total area	2.06 ± 0.09	1.86 ± 0.21	0.03
Active area	0.64 ± 0.05	0.85 ± 0.11	0.0003
Active truncated area	0.07 ± 0.01	0.11 ± 0.01	<0.0001
Suppressed area	1.42 ± 0.05	1.01 ± 0.11	<0.0001

Figure 4. Epigenetic Modifications following Multigeneration Undernutrition and Nutrient Transition

(A) Pancreatic Insulin-2 gene transcript abundance.

(B and C) Epigenetic signatures of histone modifications (relative to control, see Experimental Procedures). Areas between the rays of the radar plot were assigned as belonging to "activated" or "suppressed" parts of the epigenetic signature based on the antibodies used for IP. The Shoelace formula was then used to measure the areas of these fragments of the signature. Results were compared using t test and plotted mean ± SD.

(D and E) Areas for "active" and "suppressed" modifications before and after nutrient transition.

(F–H) Recruitment of histone modifying enzymes (F and G) and the transcription factor PDX1 (H) at rat insulin 2 promoter region.

following exposure to a dose of STZ (200 mg/kg b.w.) that rendered ≥90% Control rats (Figure 3E) diabetic (fasting blood glucose > 11 mmol/l by day 8 post-STZ). An 8-fold lower dose (25 mg/kg b.w.) offered 100% survival in Undernourished (Figure 3F) as well as R2 rats (Figure 3G) but all developing diabetes with fasting blood glucose > 11 mmol/l by day 8 from STZ injection; none of the Control animals became diabetic at this (low) dose. We observed that Undernourished and R2 rats injected with this high dose (200 mg/kg b.w.) of STZ died with hypoglycemic convulsions in 12–14 hr (Figures 3F and 3G). Serial circulating insulin measurements following STZ injection (200 mg/kg b.w.) in Undernourished and R2 rats (Figure 3H) demonstrated that the increased mortality at 200 mg/kg dose was indeed associated with a significant increase in circulating insulin within 3–6 hr after STZ injection, which resulted in hypoglycemic convulsions and death. As Undernourished and R2 rats showed 100% survival with fasting plasma glucose > 11 mmol/l by day 8, at a dose that is eight times lower than the diabetogenic dose in Control rats (200 mg/kg b.w), Undernourished and R2 rats had eight times more susceptibility to STZ-induced diabetes.

Undernourished rats also showed other markers of metabolic disorder. Elevated levels of circulating tHcy (Table S1D) is related to higher risk of coronary disease, stroke, and peripheral vascular disease and atherosclerosis in man (Zhou and Austin, 2009). Electrocardiograms (Figures S3B–S3F) revealed inverted P and T waves in R2 rats, with elevated Q and ST-segments, consistent with myocardial infarction and associated with higher early mortality and morbidity (Anderson et al., 2007), in man. A lower circulating concentration of folate (Table S1D) may itself be an atherogenic factor (Imamura et al., 2010) that could promote hyperhomocysteinemia seen in these Undernourished and R2 rats. Cardiac histology revealed multiple morphological abnormalities in R2 rats (Figure S3G) and higher cardiac tissue levels of the DNA methyl transferase *dnmt3a1* in Undernourished and R2 rats (Figure S3H), which may be associated with epigenetic silencing in cardiac tissue as well (Kotini et al., 2011).

Undernourished and R2 rat pancreas contained significantly fewer (pro-)insulin 2 gene transcripts (Figure 4A; Figures S4A and S4B), indicating that multigenerational undernutrition affected insulin gene transcription with no recovery. This may be a result of epigenetic repression of insulin gene transcription,

although active degradation of insulin gene transcripts, which have a long half-life (Gershengorn et al., 2004) of ~30–36 hr (in man), or both, is also a possibility. Indeed, the relative abundance of KMT1A, a histone-3 lysine 9-specific methyl transferase, which trimethylates H3K9me leading to suppression of gene transcription (Krauss, 2008; Rai et al., 2006), was increased in Undernourished and R2 pancreas (Figures S4C and S4D). To test whether the pro-insulin gene was epigenetically modified, we carried out chromatin immunoprecipitation (ChIP) for five different histone modifications: H3Ac, H4Ac, and H3K4me3, three modifications associated with transcriptionally activated gene promoters, and H3K9me3 and H3K20me3, two modifications associated with suppressed/silenced gene promoters. TaqMan-based real-time PCR was carried out on immunoprecipitated DNA to quantify the insulin promoter content in each of the IP fractions. Data comparing Undernourished and R2 islets to Control islets (Figure S4E) were logarithmically transformed to create radar plots of the epigenetic signature for insulin promoter (Figures 4B and 4C; Figures S4F and S4G). Areas between the rays of these radar plot were assigned as belonging to "activated" (green) or "suppressed" (orange) profiles and measured to quantify differences in overall epigenetic profiles (Figures 4D and 4E). These analyses demonstrate that epigenetic signatures leading to suppression of pro-insulin gene transcription were markedly increased (relative to Control; Figures S4E–S4H) in Undernourished rat islets and were not restored to Control levels following two generations of unrestricted access to "Control" chow (R2 rats). However, relative to the Undernourished epigenetic profiles, we observed a significant increase in activated marks ($p < 0.0001$) and a decline in suppressed marks ($p < 0.0001$) in the R2 epigenetic profile, indicating that two generations of normal feeding, significantly, but only partially, improved epigenetic repressive modifications within the insulin promoter region (Figures 4D and 4E). In order to understand the underlying molecular mechanisms leading to these histone modifications and metabolic alterations, we examined the recruitment of histone modulators and transcription factors at the insulin-2 gene promoter region. H3K9 methyl transferase KMT1A and the corepressor LSD1 were specifically recruited at the insulin gene promoter region in Undernourished and R2 rats, respectively (Figures 4F and 4G; Figures S4I–S4L). Intriguingly, such an epigenetically modified chromosomal conformation significantly diminished the spatial occupancy/recruitment of the pancreatic transcription factor PDX1, at the insulin-2 gene promoter region (Figure 4H; Figures S4I–S4L). These analyses indicate that dietary and lifestyle adaptations/choices are associated with and possibly regulatory in recruiting histone modifying enzymes at the gene promoter region. The overall chromosomal conformation seen in Undernourished rats is inhibitory to efficient binding of the transcription factor PDX1, at the insulin-2 gene promoter region.

Conclusions

The thrifty phenotype hypothesis proposes that type 2 diabetes results from the fetus and the infant having to be nutritionally thrifty, challenging the dogma that type 2 diabetes results from overnutrition in a genetically susceptible individual. The thrifty phenotype idea originated in studies that linked low birth weight with type 2 diabetes but was soon extended to the "thin-fat" body composition of the fetus and to a metabolic-endocrine profile, which suggested adaptations to tide over poor nutrition during the crucial phase of intrauterine life. Such a phenotype was advantageous if the post-natal nutrition remained poor but led to obesity, insulin resistance, and diabetes in restored food supply. Our multigenerationally undernourished rat model presents with multiple characteristics of multigenerationally deprived human populations of developing countries: low birth weight, thin-fat body composition (central adiposity), insulin resistance, characteristic dyslipidemias, and micronutrient deficiencies of methyl donors. In addition, they demonstrated heightened sensitivity to the diabetogenic doses of STZ. Molecular investigations revealed significant alterations in histone methylation, acetylation, and recruitment of histone modifying enzymes at insulin-2 gene promoter. Overall, differences in chromosomal conformation induced as a result of these modifications led to significant decrease in transcription factor PDX1 binding at the insulin-2 gene promoter. All of these may contribute to altered gene expression observed in the Undernourished rats (Figure S4M). Intriguingly, nutrient recuperation for two generations did not reverse these epigenetic modifications, but rather led to increased obesity and metabolic risk for diabetes with electrocardiographic and histological evidence for cardiovascular disease.

Current investigations failed to show any associations with genetic polymorphisms (data not shown), but further studies are warranted. Our studies have largely focused on assessing the metabolic and epigenetic changes following multigenerational undernutrition and nutrient recuperation. The thrifty genotype hypothesis (Neel, 1999) proposed that increasing prevalence of T2D among populations undergoing nutrient/lifestyle transition resulted from the selection of metabolically thrifty genes. We questioned whether genetic factors are altered during multigenerational undernutrition and whether such changes are reversed by nutrient recuperation. We initiated targeted genetic analyses in the three rat populations. Sequencing of potential SNPs in *mthfr* and *tcn2* genes (associated with cardiac, neural tube, and vitamin B_{12} defects) as well as RNA-sequencing for *Ins-2* transcripts showed no genetic polymorphisms at these loci (data not shown). Future studies involving a desired (40×) coverage through whole-genome sequencing will identify possible contributions of genetic polymorphisms toward metabolic health. Another limitation of the study is that we have assessed metabolic and epigenetic changes following multigenerational undernutrition and relatively short-term (two-generation) nutrient recuperation. Whether nutrient restoration to Undernourished animals for multiple (>2) generations may reverse adverse metabolic effects remains unknown. Another component that would be also interesting to understand is the effect of high-fat diet on Undernourished animals, which would mimic nutrient transition in today's developing countries more accurately.

Additionally, studies involving metagenome sequencing, whole-genome sequencing, and epigenetic profiling in Undernourished, Recuperation, and Control rats during nutrient transition with micronutrient (vitamin B_{12}, folate, vitamin B_6, magnesium, and vitamin D) supplementation may identify instructive mechanisms that modify our epigenomes during adaptation to a changing diet and lifestyle. The Undernourished

rat model offers unique advantages as a model of a multigenerationally undernourished population then exposed to rapid nutritional and epidemiologic transition, causing a "double burden" of disease. Our model may contribute to the development of a strategy to reduce the mismatch between early- and late-life nutrition and, therefore, facilitate development of newer strategies for diabetes prevention.

EXPERIMENTAL PROCEDURES

Animals

Undernourished rats were derived from a colony of Wistar rats (Control) by feeding a protein caloric-deficient diet (Tables S1A–S1C), as outlined in the study design (Figure 1A). Animals were housed under 12 hr day/night cycle; Control and Recuperation rats were allowed free access to standard commercial chow and water at all times. National and Institutional guidelines for the use and care of laboratory animals were followed. All procedures detailed in this study were approved by the NCCS/NTC Ethics and Animal Welfare Committees. At least 20 litters were used at each generation for propagation of this outbred colony (Figure S1A). Data represent analyses on >6 animals from 4 to 12 different litters.

Biochemical Estimations

Glucose and insulin estimations were carried as detailed in Supplemental Experimental Procedures.

Circulating biomarkers were measured on a Spectrum II Auto analyzer (Abbott Laboratories) as detailed in Supplemental Experimental Procedures.

Dual-energy X-ray absorptiometry (DXA) was carried out on age-matched males at 12, 33, or 86 weeks using Orthometrics p-DEXA scanner. Total/visceral/s.c. fat mass were measured and adiposity were calculated as amount of fat normalized to body weight at the time of measurement.

MRI was performed on age-matched rats using a Siemens 1.5 Tesla machine with 3 mm sections.

Hyperinsulinemic-euglycemic clamp studies were carried out based on the guidelines and procedures detailed by Ayala et al. (2006).

Streptozotocin (STZ), a pancreatic β-cell toxin, was reconstituted in chilled citrate buffer (pH = 4.5) prior to i.p. injection and post-STZ survival was measured as detailed in Supplemental Experimental Procedures.

Immunostaining and confocal microscopy was carried out using methods detailed in Supplemental Experimental Procedures and published earlier (Joglekar et al., 2009).

ChIP and western blotting for epigenetic modulators was carried out as detailed in Supplemental Experimental Procedures.

Quantitative real-time PCR was carried out using SybrG or TaqMan assays as detailed in Supplemental Experimental Procedures. Data are presented as "Fold over detectable" as explained elsewhere (Hardikar et al., 2014).

Statistical Analysis

Differences between groups were calculated by using one-way or two-way ANOVA and appropriate post hoc tests as described in Supplemental Experimental Procedures. SPPS, GraphPad Prism, and Jandel Scientific softwares were used to assess/plot data. A sample size of six rats in each group is sufficient to identify a difference of 27% (SD 15% of mean), with 80% power at $2p = 0.05$ between any two groups, or to identify a difference of 45% (SD 25% of mean) as statistically significant, with 80% power at $2p = 0.05$ between any two groups. Results are expressed as mean ± SEM or SD. SD scores were used to compare organ weights of Undernourished animals in comparison to the Controls. SD score = Σ [(xi – Xc)/SDc], wherein xi is individual value in the experimental (Undernourished or Recuperation) group, Xc is the mean value for the Control group, and SDc is the SD of the Control group. Difference between groups was tested by one-way ANOVA and Fisher's LSD test or Student's t test, as appropriate. Serial changes in plasma glucose and insulin concentrations were tested by paired t test or one-way ANOVA as appropriate. Computations were performed using Graphpad Prism software. Radar plots of the data representing ChIP studies were created by logarithmically transforming fold over detectable data, using shoelace formula.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.06.008.

AUTHOR CONTRIBUTIONS

A.A.H. designed, planned, carried cellular and molecular assays, data analyses, and wrote/revised the paper; S.N.S. performed animal work and biochemical assays, M.S.K. performed animal physiology studies; M.V.J. performed all epigenetic studies, immunostaining, and morphometry; W.W. and A.L. performed epigenetic studies; A.S.P. and S.K. performed clamps, DXA, and EKGs; D.S.B. performed biochemistry; A.J. performed statistics; M.R.U. conducted animal studies; A.K.R. and P.Y. performed molecular studies; R.R.B., K.A., S.G., A.C.K., A.J.J., and C.S.Y. provided infrastructure support, data analysis, and statistics. All authors read and contributed to modifications/revisions in final draft.

ACKNOWLEDGMENTS

Authors acknowledge infrastructure support through the NHMRC-CTC, JDRF Australia, Rebecca L. Cooper Foundation, and from Dr. Patwardhan (HPLC access), Dr. Ramanmurthy (animal maintenance), Dr. Sitaswad (EKG), Dr. Wani (small animal DXA), Dr. Banerjee (SGPT analysis), Dr. Rahalkar and Mr. Ghale (MRI), and Dr. K Sawaimul and Prof. Harsh Kumar (histology). Funding: A.A.H. acknowledges Australian Research Council (ARC; grant # FT110100254) and Department of Biotechnology, Government of India. M.V.J. is supported by JDRF, USA, W.W. through JDRF Australia, A.S.P. through Lady Tata Memorial fellowship, and S.N.S. through an NHMRC project grant (GNT1023060) to A.A.H.

Received: December 12, 2014
Revised: March 10, 2015
Accepted: June 9, 2015
Published: July 9, 2015

REFERENCES

Aagaard-Tillery, K.M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R., and Lane, R.H. (2008). Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J. Mol. Endocrinol. *41*, 91–102.

Aiken, C.E., and Ozanne, S.E. (2014). Transgenerational developmental programming. Hum. Reprod. Update *20*, 63–75.

Anderson, J.L., Adams, C.D., Antman, E.M., Bridges, C.R., Califf, R.M., Casey, D.E., Jr., Chavey, W.E., 2nd, Fesmire, F.M., Hochman, J.S., Levin, T.N., et al.; American College of Cardiology; American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non-ST-Elevation Myocardial Infarction); American College of Emergency Physicians; Society for Cardiovascular Angiography and Interventions; Society of Thoracic Surgeons; American Association of Cardiovascular and Pulmonary Rehabilitation; Society for Academic Emergency Medicine (2007). ACC/AHA 2007 guidelines for the management of patients with unstable angina/non-ST-Elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non-ST-Elevation Myocardial Infarction) developed in collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. J. Am. Coll. Cardiol. *50*, e1–e157.

Ayala, J.E., Bracy, D.P., McGuinness, O.P., and Wasserman, D.H. (2006). Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. Diabetes *55*, 390–397.

Echouffo-Tcheugui, J.B., and Dagogo-Jack, S. (2012). Preventing diabetes mellitus in developing countries. Nat. Rev. Endocrinol. *8*, 557–562.

Francque, S., Verrijken, A., Mertens, I., Hubens, G., Van Marck, E., Pelckmans, P., Michielsen, P., and Van Gaal, L. (2011). Visceral adiposity and insulin resistance are independent predictors of the presence of non-cirrhotic NAFLD-related portal hypertension. Int J Obes (Lond) *35*, 270–278.

Gershengorn, M.C., Hardikar, A.A., Wei, C., Geras-Raaka, E., Marcus-Samuels, B., and Raaka, B.M. (2004). Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science *306*, 2261–2264.

Goodspeed, D., Seferovic, M.D., Holland, W., Mcknight, R.A., Summers, S.A., Branch, D.W., Lane, R.H., and Aagaard, K.M. (2015). Essential nutrient supplementation prevents heritable metabolic disease in multigenerational intrauterine growth-restricted rats. FASEB J. *29*, 807–819.

Hardikar, A.A., Farr, R.J., and Joglekar, M.V. (2014). Circulating microRNAs: understanding the limits for quantitative measurement by real-time PCR. J. Am. Heart. Assoc. *3*, e000792.

Harte, A.L., Varma, M.C., Tripathi, G., McGee, K.C., Al-Daghri, N.M., Al-Attas, O.S., Sabico, S., O'Hare, J.P., Ceriello, A., Saravanan, P., et al. (2012). High fat intake leads to acute postprandial exposure to circulating endotoxin in type 2 diabetic subjects. Diabetes Care *35*, 375–382.

Imamura, A., Murakami, R., Takahashi, R., Cheng, X.W., Numaguchi, Y., Murohara, T., and Okumura, K. (2010). Low folate levels may be an atherogenic factor regardless of homocysteine levels in young healthy nonsmokers. Metabolism *59*, 728–733.

Joglekar, M.V., Joglekar, V.M., Joglekar, S.V., and Hardikar, A.A. (2009). Human fetal pancreatic insulin-producing cells proliferate in vitro. J. Endocrinol. *201*, 27–36.

Kasai, A., Gama, P., and Alvares, E.P. (2012). Protein restriction inhibits gastric cell proliferation during rat postnatal growth in parallel to ghrelin changes. Nutrition *28*, 707–712.

Kotini, A.G., Mpakali, A., and Agalioti, T. (2011). Dnmt3a1 upregulates transcription of distinct genes and targets chromosomal gene clusters for epigenetic silencing in mouse embryonic stem cells. Mol. Cell. Biol. *31*, 1577–1592.

Krauss, V. (2008). Glimpses of evolution: heterochromatic histone H3K9 methyltransferases left its marks behind. Genetica *133*, 93–106.

Murray, M., Zaluzny, L., and Farrell, G.C. (1986). Drug metabolism in cirrhosis. Selective changes in cytochrome P-450 isozymes in the choline-deficient rat model. Biochem. Pharmacol. *35*, 1817–1824.

Neel, J.V. (1999). The "thrifty genotype" in 1998. Nutr. Rev. *57*, S2–S9.

Ng, S.F., Lin, R.C., Laybutt, D.R., Barres, R., Owens, J.A., and Morris, M.J. (2010). Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. Nature *467*, 963–966.

Rai, K., Nadauld, L.D., Chidester, S., Manos, E.J., James, S.R., Karpf, A.R., Cairns, B.R., and Jones, D.A. (2006). Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. Mol. Cell. Biol. *26*, 7077–7085.

Smith, W.W., Brecher, G., Budd, R.A., and Fred, S. (1966). Effects of bacterial endotoxin on the occurrence of spleen colonies in irradiated mice. Radiat. Res. *27*, 369–374.

Stewart, R.J., Sheppard, H., Preece, R., and Waterlow, J.C. (1980). The effect of rehabilitation at different stages of development of rats marginally malnourished for ten to twelve generations. Br. J. Nutr. *43*, 403–412.

Suter, M.A., Chen, A., Burdine, M.S., Choudhury, M., Harris, R.A., Lane, R.H., Friedman, J.E., Grove, K.L., Tackett, A.J., and Aagaard, K.M. (2012). A maternal high-fat diet modulates fetal SIRT1 histone and protein deacetylase activity in nonhuman primates. FASEB J. *26*, 5106–5114.

Wells, J.C. (2006). The evolution of human fatness and susceptibility to obesity: an ethological approach. Biol. Rev. Camb. Philos. Soc. *81*, 183–205.

Yajnik, C.S. (2004). Early life origins of insulin resistance and type 2 diabetes in India and other Asian countries. J. Nutr. *134*, 205–210.

Yajnik, C.S., and Deshmukh, U.S. (2012). Fetal programming: maternal nutrition and role of one-carbon metabolism. Rev. Endocr. Metab. Disord. *13*, 121–127.

Yajnik, C.S., Fall, C.H., Vaidya, U., Pandit, A.N., Bavdekar, A., Bhat, D.S., Osmond, C., Hales, C.N., and Barker, D.J. (1995). Fetal growth and glucose and insulin metabolism in four-year-old Indian children. Diabet. Med. *12*, 330–336.

Yajnik, C.S., Fall, C.H., Coyaji, K.J., Hirve, S.S., Rao, S., Barker, D.J., Joglekar, C., and Kellingray, S. (2003). Neonatal anthropometry: the thin-fat Indian baby. The Pune Maternal Nutrition Study. Int. J. Obes. Relat. Metab. Disord. *27*, 173–180.

Yajnik, C.S., Deshpande, S.S., Jackson, A.A., Refsum, H., Rao, S., Fisher, D.J., Bhat, D.S., Naik, S.S., Coyaji, K.J., Joglekar, C.V., et al. (2008). Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia *51*, 29–38.

Yajnik, C.S., Chandak, G.R., Joglekar, C., Katre, P., Bhat, D.S., Singh, S.N., Janipalli, C.S., Refsum, H., Krishnaveni, G., Veena, S., et al. (2014). Maternal homocysteine in pregnancy and offspring birthweight: epidemiological associations and Mendelian randomization analysis. Int. J. Epidemiol. *43*, 1487–1497.

Zhou, J., and Austin, R.C. (2009). Contributions of hyperhomocysteinemia to atherosclerosis: Causal relationship and potential mechanisms. Biofactors *35*, 120–129.