Transgenerational Inheritance of Glucose Intolerance in a Mouse Model of Neonatal Overnutrition

Thais Pentinat, Marta Ramon-Krauel, Judith Cebria, Ruben Diaz, and Josep C. Jimenez-Chillaron

Research, Fundacio Sant Joan de Deu (T.P., J.C., J.C.J.-C.), and Endocrine Division, Hospital Sant Joan de Deu (M.R.-K., R.D., J.C.J.-C.), Barcelona 08950, Spain; and Endocrine Division (R.D., J.C.J.-C.), Children's Hospital Boston and Harvard Medical School, Boston, Massachusetts 02115

Epidemiological and clinical data show that rapid weight gain early in life is strongly associated with several components of the metabolic syndrome. Strikingly, abnormal growth rates in early life can additionally influence diabetes risk in subsequent generations. Here we aim to study whether neonatal overgrowth induces diabetes in offspring and grand-offspring of affected individuals using a mouse model of neonatal overfeeding. We induced neonatal overgrowth (ON-F0) by culling offspring to four pups per dam during lactation. By age 4 months, ON-F0 mice developed many features of the metabolic syndrome, including obesity, insulin resistance, and glucose intolerance. We then studied whether male offspring (ON-F1) and grand-offspring (ON-F2) of ON-F0 male mice, which were not overfed during lactation, developed features of the metabolic syndrome with aging. ON-F1 mice developed fed and fasting hyperinsulimemia, hypertryglyceridemia, insulin resistance, and glucose intolerance, but not obesity, by age 4 months. In contrast, ON-F2 male mice showed a more moderate phenotype and only developed fasting hyperglycemia and glucose intolerance by age 4 months. Impaired glucose tolerance in ON-F1 and ON-F2 mice appeared to be accounted for primarily by peripheral insulin resistance, because beta-cell function remained normal or even increased in these cohorts. Nutritional challenges occurring during sensitive periods of development may have adverse metabolic consequences well beyond the lifespan of affected individuals and manifest in subsequent generations. Transgenerational progression of metabolic phenotypes through the male lineage supports a potential role for epigenetic mechanisms in mediating these effects. **(*Endocrinology* 151: 5617–5623, 2010)**

Epidemiological and clinical data show that rapid weight gain early in life is strongly associated with several components of the metabolic syndrome, including cardiovascular disease, type 2 diabetes, and obesity (1–5). Overfeeding is the primary mediator of rapid neonatal weight gain (1). Human data are further supported by experimental models: neonatal overfeeding in rats promotes rapid weight gain and programs many features of the metabolic syndrome later in life (6, 7). Importantly, in these experimental paradigms, animals are maintained on a controlled standard chow diet from weaning onward, demonstrating that early overfeeding/overgrowth *per se* increases risk of late-onset chronic diseases.

In addition, recent epidemiological evidence suggests that abnormal nutrition in early life can influence diabetes risk in subsequent generations (8). It has been shown that augmented food availability during the slow prepubertal growth period in grandfathers increases the risk of cardiovascular and diabetes-related deaths in their grandsons (9–12). The authors suggest that there exists a sex-specific male-lineage transgenerational inheritance of disease risk. While mechanisms are unclear, and genetic contributions from the Y chromosome cannot completely be ruled out, epigenetic mechanisms might explain such transgenerational effects. Nevertheless, better understanding of mechanisms linking neonatal growth with late-onset disease,

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Abbreviations: F0, Parental generation; F1, first generation offspring; F2, second generation offspring; HOMA-IR, homeostatic model assessment–insulin resistance; ipGTT, intraperitoneal glucose tolerance tests; NEFA, nonesterified fatty acids; ON, overnutrition.

and transgenerational effects, is curtailed, in part, by lack of appropriate animal models.

We created a mouse model of neonatal overfeeding and accelerated early growth rate (ON-F0) that develops insulin resistance, glucose intolerance, and diabetes by age 6 months. The aim of this work was to explore whether the risk of obesity, insulin resistance, and other features of the metabolic syndrome is carried to their offspring and grand-offspring via the paternal lineage. We show, for the first time, that male offspring and grand-offspring of ON-F0 male mice also develop insulin resistance and glucose intolerance with aging. Transgenerational progression of metabolic phenotypes through the paternal lineage supports a potential role for epigenetic mechanisms in mediating these effects.

Materials and Methods

Animal care and experimental design

Protocols were approved by the Universitat de Barcelona Animal Care and Use Committee. ICR mouse strain (ICR-CD1, Harlan Laboratories, Italy) was chosen for this study based on its fast somatic growth, especially during the neonatal period. Besides, ICR mice have been previously shown to be a valid model to understand the association between neonatal growth and adult metabolism (5, 17, 18). Eight-week-old virgin females were mated with not sibling males. Upon confirmation of pregnancy, females were housed individually with *ad lib* access to standard chow (2014 Tekland Global, Harlan Iberica, Barcelona, Spain). After delivery, litter size was adjusted to eight pups (control group, C) or four pups per dam (overnutrition group, ON). Both C and ON offspring are designated as the parental generation, F0 (Fig. 2A). F0 pups were nursed freely and weaned at 3 weeks onto standard chow, provided *ad libitum*. At weaning, C and ON mice were housed in groups of six mice per cage. C and ON males from the F0 generation were mated at age 3 months with external control virgin females, provided by the vendor (Harlan), to generate the first generation-offspring, F1 (Fig. 2A). All females had the same average weight and age (8–10 wk) to avoid potential metabolic biases, due to maternal effects, in the offspring (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Likewise, male breeders for each crossing were randomly selected to guarantee an unbiased representation for each experimental group (Supplemental Fig. 1). During the mating process we kept one male with one single female. After confirmation of pregnancy by vaginal plug, the male was removed from the cage and the female was maintained individually throughout gestation. At birth, all litters are adjusted to eight pups per dam. Thus, contrary to the parental generation, ON-F1 pups are not neonatally overfed compared with their matched controls. We next repeated the breeding protocol, by using C-F1 and ON-F1 males, to obtain the second-generation offspring, F2 (Fig. 2A). Likewise, all litters are equalized to 8 pups per dam to match normal nutrition during the neonatal period. At weaning all mice have free access to standard chow.

In this study, we focus on the metabolic analysis of males only, because paternally-induced transgenerational effects should be mediated, primarily, by epigenetic mechanisms. In contrast, maternally-induced transgenerational effects might be mediated by a complex interplay between metabolic, mitochondrial and epigenetic modifications.

Neonatal food intake was determined in 4-d-old neonates as described (13). Briefly, at 0900 the whole litter was isolated from the mother, and neonates were fasted for three hours. To avoid hypothermia, neonates were maintained on a thermal electric blanket during this period. After the 3-hour fasting, mice were weighed accurately on a high precision scale and reintroduced with the mother for 1 h. After the 1-h refeeding period, neonates were weighed again. Differences in body weight are a good estimate of food intake. Adult food intake was recorded from 4-month-old individual mice for five consecutive days. Food was weighed every 24 h, and the weight difference is a measure of daily food intake. Cumulative food intake is presented as the progressive accumulation of food consumed over the course of 5 d.

Epididymal fat mass assessment

Fat mass was determined in 5-month-old mice. Epididymal fat depots were dissected and fat mass calculated as a percentage of wet tissue per whole body weight.

In vivo metabolic testing

Intraperitoneal glucose (2 g/kg weight) tolerance tests (ipGTT) were performed in unrestrained conscious mice after a 12-h fast. Insulin release was assessed during the ipGTT as follows: $\Delta Insulin_{30\text{-}0\ min}/\ \Delta Glucose_{30\text{-}0\ min}$. Insulin sensitivity was determined by homeostatic model assessment–insulin resistance (HOMA-IR), as described (14, 15). HOMA is calculated by using both fasting glucose and insulin as follows: HOMA-IR = Glucose × Insulin/405, where glucose is given in mg/dl and insulin is given in μU/ml.

Serum analysis

Insulin was measured by ELISA (Millipore, Spain). Blood glucose was measured with a Glucose Meter Elite (Menarini, Barcelona, Spain). Triglycerides, glycerol, and nonesterified fatty acids (NEFA) were measured using colorimetric methods on 2-μl serum samples (BioVision, Madrid, Spain).

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using a two-tailed *t* test or a one-way ANOVA as indicated (IBM SPSS Statistics 19, Madrid, Spain). A P value <0.05 was considered significant.

Results

Neonatal accelerated growth programs metabolic syndrome in the adult

We show that neonatal overfeeding in ON-F0 male mice (Fig. 1A) led to accelerated postnatal weight gain during the first weeks of life (Fig. 1B). By age 2 weeks, ON-F0 mice were already heavier than controls (Fig. 1B); differences in body weight persisted until adulthood ($P = 0.0002$) (Fig. 1C), despite normalization of food intake by age 4 months (Fig. 1D). Likewise, ON-F0 mice showed

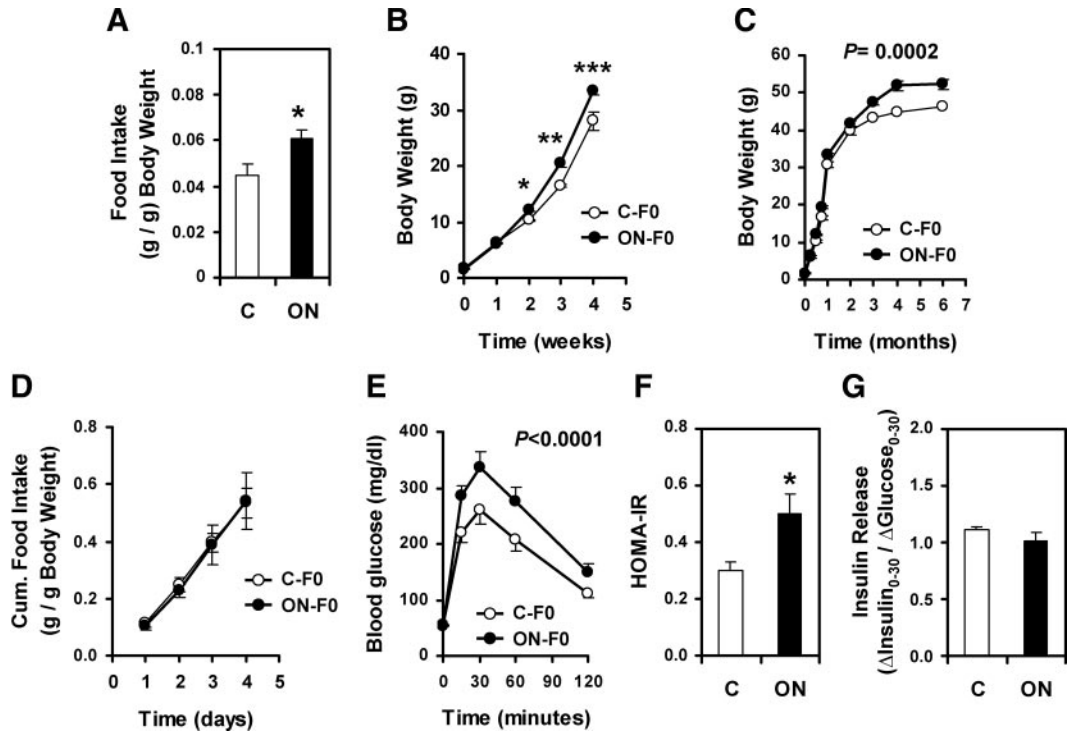

FIG. 1. Physiological characterization of ON-F0 male mice. A, Neonatal food intake on 4-d-old mice. n ≥ 6 mice per group. B, Early postnatal growth from birth to age 4 weeks. n ≥ 6 mice per group. C, Body weight from birth until age 6 months. n ≥ 20 mice per group. D, Cumulative food intake in 4-month-old male mice over the course of 1 week. n ≥ 6 mice per group. E, Glucose tolerance test (2 g glucose/kg body weight) was performed in unrestrained 4-month-old male mice after an overnight fast. n ≥ 14 mice per group. F, HOMA-IR. Insulin sensitivity was assessed as follows: HOMA-IR = [fasting insulin (mU/liter) × fasting glucose (mg/dl)]/405. n ≥ 14 mice per group. G, Insulin released during the glucose tolerance test. Insulin release was measured as the ratio between insulin excursion from 0 to 30 min/ glucose excursion from 0 to 30 min. n ≥ 8 mice per group. Results in all panels are expressed as mean ± SEM. *, $P < 0.05$ *vs.* Control; ***, $P < 0.001$ *vs.* Control (Student's *t* test). Statistical analysis between groups in panels C and E was evaluated by one-way ANOVA and included in the graph. $P < 0.05$ was considered significant.

increased epididymal fat mass (Table 1). As expected, 4-month-old ON-F0 mice developed hypertriglyceridemia, fed and fasting hyperinsulinemia (Table 1), glucose intolerance ($P < 0.0001$) (Fig. 1E), and insulin resistance (Fig. 1F). Because impaired glucose tolerance may result from insulin resistance and/or impaired insulin secretion, we further determined *in vivo* β-cell function and demonstrated that glucose-stimulated insulin release during the glucose tolerance was preserved in ON-F0 mice compared with controls (Fig. 1G).

Transgenerational effects of neonatal overfeeding

Next, we explored whether ON-F0 associated phenotypes are inherited by subsequent generations through the paternal lineage (Fig. 2A). Birth weight, sex distribution, litter size, and length of gestation of ON-F1 and ON-F2

TABLE 1. Growth data, glucose homeostasis, and hormonal data in 4-month-old male mice from F0, F1 and F2 generation offspring

	F0		F1		F2	
	C	ON	C	ON	C	ON
Epididymal fat mass (% body weight)	1.39 ± 0.18 (30)	2.67 ± 0.23** (22)	0.76 ± 0.09 (28)	0.52 ± 0.67* (29)	1.48 ± 0.22 (21)	1.69 ± 0.43 (12)
Glucose, random fed (mg/dl)	117.00 ± 2.87 (38)	158.90 ± 16.06** (24)	131.30 ± 4.31 (26)	115.90 ± 4.10 (26)	122.90 ± 4.59 (23)	129.10 ± 3.43 (13)
Glucose, fasted (mg/dl)	55.40 ± 2.31 (21)	56.40 ± 3.48 (22)	53.70 ± 2.10 (21)	86.30 ± 7.28* (11)	52.70 ± 2.32 (16)	64.90 ± 5.96* (13)
Insulin, random fed (ng/ml)	1.42 ± 0.30 (28)	6.06 ± 1.40*** (23)	1.57 ± 0.30 (14)	3.10 ± 0.77* (15)	0.87 ± 0.13 (23)	1.27 ± 0.28 (12)
Insulin, fasted (ng/ml)	0.17 ± 0.01 (13)	0.34 ± 0.12* (13)	0.27 ± 0.02 (11)	0.45 ± 0.06** (6)	0.14 ± 0.01 (10)	0.28 ± 0.08 (10)
TAG (nM)	4.80 ± 1.03 (7)	16.30 ± 2.34*** (8)	4.97 ± 0.79 (8)	9.03 ± 0.79* (8)	4.58 ± 0.65 (11)	3.39 ± 0.19 (11)
NEFA (nM)	13.46 ± 0.29 (8)	14.50 ± 1.26 (8)	14.02 ± 1.73 (8)	15.77 ± 1.70 (8)	13.45 ± 0.37 (12)	13.47 ± 0.46 (5)

Results are expressed as mean ± SEM.

*, $P < 0.05$ *vs.* Control; **, $P < 0.01$ *vs.* Control; ***, $P < 0.001$ *vs.* Control (Student's *t* test). n value for each group is specified in the brackets.

mice were similar to controls (not shown). Likewise, neonatal food intake (not shown) and early postnatal growth of ON-F1 and ON-F2 male mice was normal when compared with control mice (Fig. 2B). Also, body weight of ON-F1 and ON-F2 mice was similar to controls until age 6 months (Fig. 2C).

Strikingly, some metabolic abnormalities in ON-F0 mice were inherited by subsequent generations. Indeed, 4-month-old ON-F1 male mice developed moderate fasting hyperglycemia, hyperinsulinemia, and hypertriglyceridemia (Table 1). Likewise, ON-F1 mice showed insulin resistance and mild impaired glucose tolerance ($P < 0.03$) when compared with controls (Fig. 2, D and E). Insulin release was actually increased in ON-F1 mice when compared with controls (Fig. 2F). These data suggest that, as in ON-F0 mice, glucose intolerance arises predominantly as a consequence of insulin resistance in ON-F1 mice. NEFA remained normal when compared with control mice (Table 1). Finally, and contrary to what happened to ON-F0 mice, fat mass was significantly reduced in ON-F1 mice (Table 1). Thus, we show that many, but not all, metabolic disturbances occurring in ON-F0 mice are inherited by the F1.

We next asked whether ON-F0 associated phenotypes are still present in the F2. At 4 months of age, ON-F2 male mice still exhibited mild fasting hyperglycemia (Table 1) and impaired glucose tolerance ($P < 0.02$) (Fig. 2D). Similarly, ON-F2 male mice showed a nonstatistical trend for increased insulin resistance (Fig. 2E). By contrast, ON-F2 male mice exhibited normal serum triglycerides, NEFA, and insulin (Table 1). Likewise, fat mass and β-cell function remained normal compared with controls (Table 1, Fig. 2F). Thus, we show that only a small fraction of metabolic abnormalities occurring in ON-F0 and ON-F1 mice are transmitted to the F2.

Discussion

We have developed a mouse model of neonatal overnutrition and accelerated growth rate that develops glucose intolerance, obesity, and insulin resistance with aging. In agreement, human studies show that accelerated growth rate during infancy may lead to childhood obesity and increases risk of diabetes in adulthood (4). Moreover, neonatal overfeeding in rats also leads to rapid weight gain and development of obesity and diabetes in the adult (6, 7, 16). Hence, both human and animal data clearly suggest that growth trajectories during early life may influence adult metabolism and might be a good predictor for later risk of chronic disease (4, 5). In accord, we had previously described that postnatal slow growth rate, due to reduced caloric intake, results into the opposite phenotype: adult mice that exhibited slow neonatal growth rate were lean, insulin sensitive, hypoinsulinemic, and have some improvement on glucose tolerance compared with control mice (5, 17, 18). In conclusion, these data suggest that control of neonatal feeding and, hence, neonatal growth are critical mediators of adult health and disease.

Recent data suggest that neonatal and/or childhood overfeeding may additionally have consequences for subsequent generations: augmented food availability during the prepubertal growth period in men predisposes to diabetes and diabetes-related death in their grandsons (10). In agreement, here we demonstrate that neonatal overfeeding predisposes to glucose intolerance and fasting hyperglycemia, not only in the exposed individuals but also their offspring and their grand-offspring. Strikingly, we show that these neonatally-induced diabetes-related phenotypes can be inherited through the male lineage. While it is well known that the mother's metabolism strongly influences her offspring's metabolism (maternal effects) (8, 19), literature describing paternal effects is scant, with only a few examples in animal models (20–22) and humans (10). These data, including ours, are of clinical relevance, because they suggest that paternal history may have a more profound influence on offspring metabolism than previously thought.

Mechanistically, inheritance of environmentally-induced phenotypes through the paternal lineage is likely due to epigenetic modifications residing in cells from the germ line (23, 24). In this regard, it has been shown that nutrition and other environmental cues early in life may modify the epigenome, including DNA methylation and histone modifications, in both somatic and germ cells (16, 23, 25–27). For example, and relevant to our model, it has been recently described that neonatal overfeeding may change patterns of DNA methylation in the proximal promoter of the anorexigenic hypothalamic gene POMC (16). This results in lack of POMC upregulation in response to leptin and insulin, which might explain, in part, obesity-associated hyperphagia in this rat model.

Thus, taking together all previous observations, here we propose that in our model early overfeeding might cause permanent alterations in both somatic and germ cells, in part through epigenetic modifications. Adaptations in somatic cells may explain diabetic phenotypes in F0 mice, whereas modifications in germ cells might provide the basis for the transgenerational effects. Alternatively, it might be also possible that paternal obesity *per se* (or obesity-associated metabolism) induces, indirectly, modifications in sperm that are, in turn, transmitted to the following generation. As a matter of fact, ON-F0 male breeders are actually heavier than controls

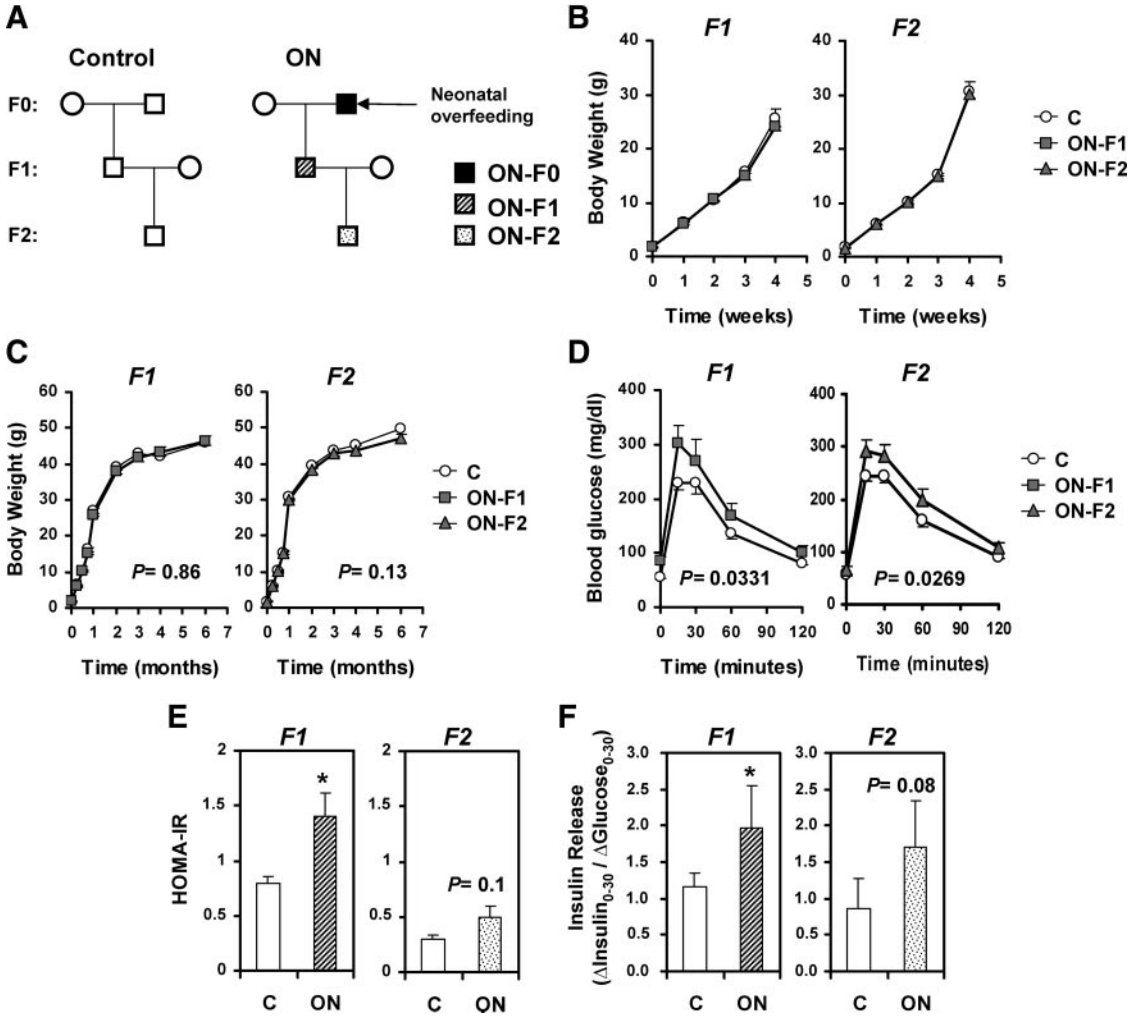

FIG. 2. Physiological characterization of ON-F1 and ON-F2 male mice. A, Experimental design, including the breeding scheme for first (F1) and second-generation (F2) offspring. Circles designate females and squares designate males as indicated in the *Materials and Methods* section. Metabolic analysis was performed in males only. B, Early postnatal growth from birth to age 4 weeks. 15 ≥ 6 mice per group. C, Body weight from birth until age 6 months. n ≥ 20 mice per group. D, Glucose tolerance test (2 g glucose/kg body weight) was performed in unrestrained 4-month-old male mice after an overnight fast. n ≥ 14 mice per group. E, HOMA-IR. Insulin sensitivity was assessed by the homeostatic model assessment as described in the *Materials and Methods* section. n ≥ 14 mice per group. F, Insulin released during the glucose tolerance test. n ≥ 8 mice per group. Results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* Control (Student's *t* test). In panels C and D, statistical analysis between groups was evaluated by ANOVA and results included in the graphs. $P < 0.05$ was considered significant.

(Supplemental Fig. 1). While we cannot distinguish between these two potential options, it will be interesting to design an experiment where neonatal overnutrition does not result in adult obesity and ask whether lean ON-F0 mice also transmit metabolic phenotypes to the following generation.

Physiologically, impaired glucose tolerance in ON-F0 mice might be primarily attributed to peripheral insulin resistance rather than impaired β-cell function. Indeed, insulin release during an ip glucose tolerance is normal in ON-F0 mice, thus indicating that β-cells are still able to partially compensate for the developing insulin resistance. Likewise, impaired glucose tolerance in ON-F1 and ON-F2 mice might be accounted for primarily by peripheral insulin resistance. In agreement, fasting hyperglycemia in ON-F1 and ON-F2 male mice might suggest uncontrolled hepatic gluconeogenesis, probably due to liver insulin resistance. This possibility will be further investigated.

Of note, despite these similar physiological trends across all three generations, inheritance of phenotypes is heterogeneous and does not equally involve all alterations described in ON-F0 mice: Thus, ON-F1 male mice develop insulin resistance, hypertriglyceridemia, elevated fasting glucose, impaired glucose tolerance, and a paradoxical reduction of fat mass, as assessed by epididymal fat content. On the other hand, ON-F2 mice have a milder phenotype than ON-F1 mice, characterized by moderate fasting hyperglycemia and impaired glucose tolerance only. Thus, we report that metabolic dysregulation is strongly reduced in second-generation offspring. Transgenerational weakening of phenotypes has

been previously reported in other animal models (22, 28, 29). As we have already discussed, progressive weakening of phenotypes indicates that these effects are likely mediated by epigenetic modifications rather than by changes in DNA sequence, that stay stable across generations (8).

Conclusion

Here we show, for the first time, that male offspring and grand-offspring from neonatally over nourished male mice develop glucose intolerance by age 4–6 months. Transgenerational inheritance of metabolic dysfunction through the paternal lineage suggests that phenotypes are transmitted through the gametes, likely due to nutritionally-induced epigenetic modifications. Importantly, metabolic phenotypes fade away as generations fall apart from the original environmental cue, thus reinforcing the idea that transgenerational phenotypic progression occurs through nongenomic mechanisms. In sum, nutritional challenges occurring during sensitive periods of development, such as the early neonatal period, may have adverse metabolic consequences well beyond the lifespan of affected individuals and manifest in subsequent generations.

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Address all correspondence and requests for reprints to: Josep C. Jimenez-Chillaron or Ruben Diaz, Fundacio Sant Joan de Deu, c/ Santa Rosa 39-57, 4ª Planta, Esplugues de Llobregat, Barcelona, Spain, 08950. E-mail: jjimenezc@fsjd.org or rdiaz@hsjdbcn.es.

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