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Transgenerational effects of obesogens and the obesity epidemic

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In the last three decades there has been a dramatic, worldwide increase in the incidence of obesity, particularly in Western societies. This trend has required a significant economic investment to treat obesity-related disorders such as type 2 diabetes, cardiovascular disease, and non-alcoholic fatty liver disease. There is an urgent need to understand the factors that contribute to this increase in obesity in order to find new tools that will improve quality of life in affected individuals and to avoid the propagation of obesity to future generations. Endocrine disrupting chemicals have become an important piece of the obesity epidemic puzzle but little is known about the mechanism underlying their effects. In this commentary, we highlight recent work showing that the consequences of ancestral exposure to obesogenic chemicals results in the transmission of obesity-related phenotypes through at least three generations.

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The obesity epidemic

Obesity has become a worldwide epidemic not only in developed countries, but also in developing nations [1,2]. There are other metabolic risk factors associated with obesity such as insulin resistance, hypertension or non-alcoholic fatty liver, that are strongly correlated with the development of cardiovascular disease and diabetes [3]. In the last decade, this increase in the incidence of obesity and obesity-related pathologies not only has affected adults but also young children [2,4–6].

It is widely accepted that a major explanation for the increase in fat mass is an impaired balance between

energy intake and energy expenditure — the ‘calories in–calories out’, or thermodynamic model [7]. However, several other factors are now considered to be obesity enhancers in addition to the proposed overconsumption of food and a sedentary lifestyle; these include maternal smoking, stress, excessive consumption of alcohol and genetics [8–12]. There is a growing body of evidence showing that exposure to endocrine disrupting chemicals (EDCs) can adversely affect our health [13,14]. EDCs are defined as exogenous chemicals, or mixtures of chemicals that interfere with any aspect of hormone action [15]. There is abundant evidence that both wildlife and humans are widely exposed to EDCs resulting in a broad spectrum of adverse consequences [13,14,16]. It has been recently demonstrated that animals living in close proximity to human populations, such as pets, feral rodents, and laboratory animals have shown an overall increase in body weight in the last decades [17]. The fact that some of these animals are housed in highly controlled conditions (e.g. laboratories) indicates that the overall weight gain of human-associated animal species cannot solely be ascribed to the effects of unhealthy human food waste and lifestyle. The types of food given to these laboratory animals (sterile vs. non-sterile) and the source of their components (grains, alfalfa, among others, used in the pellets) may be important factors contributing to the increasing rates in body weight since they condition the intestinal microbiome, which has been associated with the development of obesity [18,19]. Furthermore, the components of various materials used to house the animals (plastic cages, plastic water bottles, bedding, among others) may also be important factors that are commonly not taken into consideration when designing new experiments. These bio-indicators suggest that there might be other environmental factors that are affecting the development of obesity at the community and population levels. Human epidemiological studies relating EDC exposure to the development of obesity are limited, but several positive associations have been noted in recent years [20,21,22,23]. There is abundant evidence from studies in laboratory animals and in cell culture that a variety of EDCs can lead to increased adipogenesis and weight gain [24–31,32,33,34,35,36,37–39].

Transgenerational effects of EDCs and obesogens

Obesogens are functionally defined as chemicals (some of which are EDCs) that increase lipid accumulation and obesity by acting directly to induce lipid accumulation, adipocyte hypertrophy or hyperplasia or indirectly to alter

metabolism, or the regulation of appetite and satiety [40]. A number of obesogenic chemicals have been identified in the past 10 years, supporting the idea that exposure to obesogenic EDCs may be playing an unsuspected role in the obesity epidemic. These include estrogenic EDCs such as diethylstilbestrol (DES) [37], bisphenol A (BPA) [31,38] and dichlorodiphenyltrichloroethane (DDT) [41**]. Other classes of chemicals include organotins such as tributyltin (TBT) [26,32], perfluorooctanoates [35] and phthalates [33*,34,36**], all of which have been shown to be obesogenic in animals. High levels of several persistent organic pollutants (e.g. dichlorodiphenyldichloroethylene [DDE], hexachlorobenzene (HCB), polybrominated diphenylethers) were positively associated with obesity in humans [42] and urinary phthalate levels were correlated with increased waist diameter and insulin resistance in humans [43,44].

Previous work from our laboratory demonstrated that TBT induces adipogenesis, *in vivo* and *in vitro*, by activating the peroxisome proliferator-activated receptor gamma (PPAR γ) and the retinoid X receptor (RXR), key regulators of adipogenesis, and predisposing multipotent mesenchymal stem cells (MSCs) to differentiate into adipocytes [26,28,45]. We recently showed that exposure of pregnant F0 mice to environmentally relevant doses of TBT leads to the development of obesity in subsequent generations [32]. In our model, pregnant females (F0) were exposed to 5.42 nM, 54.2 nM or 542 nM of TBT in the drinking water. The no observable adverse effect level (NOAEL) for TBT (based on its immunotoxicity) in rodents is 25 $\mu\text{g}/\text{kg}/\text{day}$ [46] and the doses used in our study correspond to 50-fold lower, 5 fold lower and 2-fold higher than the NOAEL, respectively. We found increased adiposity in F1, F2 and F3 generations. This is notable because, while the F1 animals were exposed *in utero* and the F2 animals were exposed as germ cells in the F1 generation, the F3 generation did not receive any exposure to TBT [32]. We noted increased adipose depot weight, adipocyte size and number in three different white adipose depots (2 visceral depots, and 1 subcutaneous depot), the development of non-alcoholic fatty liver disease (NAFLD) phenotype and the reprogramming of mesenchymal stem cells (MSCs) toward the adipogenic lineage at the expense of the bone lineage.

There is relatively little known about the extent of human exposure to TBT. Exposure is likely to occur through consumption of seafood contaminated with TBT and via the use of TBT and related chemicals as fungicides and miticides on food crops, in wood treatments, industrial water systems, textiles, and via leaching of organotin-stabilized PVC from water pipes, food wrap and other plastics [47]. Organotins have also been found in appreciable levels in house dust, suggesting that much of the population is exposed on a regular basis [48]. A

recent study showed a positive correlation between placental TBT levels and weight gain in male infants at 3 months of age [22]. Therefore, TBT exposure is associated with weight gain both in animals and in humans and future human biomonitoring studies are justified and relevant to the obesity epidemic.

Obesity in humans is characterized by the accumulation of fat in the body leading to a body mass index of >30 kg per meter of height squared. However, fat distribution all over the body varies depending on the physiology of the individual, contributing to the development of certain diseases associated with the increase in fat accumulation [40,49]. Although the mechanism determining this pattern between different individuals is still not fully understood, it is well known that the risk of health consequences associated with obesity differs depending on where the fat is located. For instance, subcutaneous adipose tissue captures the excess of free fatty acids and glycerol and transforms them in triglycerides, which is generally considered to be an adaptive process. When the subcutaneous depots exceed their storage capacity it is the visceral cavity that captures the extra free fatty acids [50]. An increase in visceral adipose tissue is highly correlated with increased mortality and risk for disorders such as diabetes, hyperlipidemia, hypertension, cardiovascular disease, insulin resistance and inflammatory diseases compared with increased subcutaneous fat accumulation [51]. We have shown that the ancestral *in utero* exposure to low doses of TBT leads to an increase in adipose tissue in at least three generations in mice [32] but it is currently uncertain what molecular mechanisms underlie this phenotype. One possibility is that the bias in the fate of MSCs toward the adipocyte lineage leads animals to accumulate more fat from the diet. MSCs are pluripotent stem cells that can differentiate toward a variety of lineages including adipogenic, osteogenic or cartilage lineages depending on the stimuli they receive [52]; therefore, it is plausible that the animals are predisposed to possess more fat cells and store more ingested calories as fat. The available evidence suggests that TBT-treated animals do not eat more than untreated animals, which would defy the conventional wisdom about obesity being the simple result of caloric imbalance.

Mechanism of transgenerational effects of EDCs on obesity

Our observations indicate that prenatal TBT exposure elicited changes in the germ cells of F1 fetuses that were transmitted to subsequent generations [32]. The experimental design (multiple independently treated dams, not breeding siblings) makes it unlikely that the changes are genetic in origin. Rather, this may be an example of a maternal programming event that permanently alters stem cell fate leading to a reproducible adult phenotype. We currently know very little about how EDC exposure can cause heritable, transgenerational changes in the

genome that alter the fate of MSCs, or predispose hepatocytes to accumulate lipids. Skinner and colleagues independently showed that a variety of chemicals can cause transgenerational effects in rats, including obesity. These include a mixed hydrocarbon mixture (jet fuel JP-8) [53^{*}] and common plastic components such as BPA, diethylhexyl phthalate and dibutyl phthalate [36^{**}]. Perhaps most importantly, they showed that the quintessential EDC, DDT, lead to a striking increase in obesity in F3 rats derived from F0 exposure [41]. They also identified the first examples of transgenerationally inherited sperm epigenetic mutations (epimutations) that might underlie the observed disease outcomes [54^{**}]. It is an open question whether the effects of DDT are mediated directly via its action through estrogen receptors, or whether its primary metabolite DDE (an anti-androgen) is the causal factor.

Are transgenerational effects epigenetic?

It is generally accepted that maternal diet and prenatal nutrition can have transgenerational effects [55^{*},56^{*}] but there is dispute about whether these changes are epigenetic and whether epimutations are important for human disease [57^{*},58]. There are multiple epigenetic components (DNA methylation, histone modifications, non-coding RNA, among others) that participate in the modulation of gene expression. The DNA methylation (5' methylation or 5' hydroxymethylation of cytosines) and histone modifications patterns are determined in early stages of embryonic development in mammals [59]. During this period, DNA methylation marks are erased from the genome to be subsequently remethylated in later stages to ensure the correct imprinting reprogramming (expression of certain genes in a parent-of-origin manner, independent of Mendelian inheritance) [60]. During the period of time the genome is demethylated, external factors may contribute to a new epigenetic patterning of the DNA, altering the imprinting pattern of the genome and, therefore, modifying the gene expression of target genes. There is a growing consensus that effects of EDCs on maternal programming events in animals are likely to be caused by epigenetic changes [61–63], but the nature of these changes remains elusive. An obvious possibility is that changes in DNA methylation might be linked with transgenerational changes in gene expression [62]. Indeed, exposure of laboratory rodents to EDCs can modify the methylation pattern of cytosines at metastable alleles (e.g. *agouti*) [64] leading to phenotypes such as obesity and a recent study identified altered DNA methylation at metastable alleles in human infants that were correlated with maternal methyl donor levels resulting from nutritional factors [65^{**}]. Therefore, it is possible that EDC-exposed human fetuses might also exhibit changes in the epigenome. Skinner and colleagues identified candidate consensus motifs, such as zinc finger binding regions and guanine quadruplexes, in differential DNA methylation regions in

sperm from animals exhibiting transgenerational inheritance of disease and particular phenotypes, including obesity [54]. The alteration of the methylation pattern of these motifs will modify the capability of DNA methyltransferases and transcription factors to bind to these areas, which ultimately will modify the methylation pattern of those regions of the genome and the expression of the affected genes. It will be of great interest to determine whether and which of these modifications are causally associated with the phenotypes observed.

Another candidate epimutation is 5' hydroxymethylation at cytosines (5-hmeC). Numerous recent studies have linked 5-hmeC with stem cell differentiation and these modified residues appear to frequently be associated with the promoters of key genes expressed in stem cells that modulate development and differentiation [66–70]. 5-hmeC has also been shown to be an important intermediate in the DNA demethylation process and it is often the case that 5-hmeC are produced in regions where demethylation is occurring [71,72]. Thus, these 2 processes appear to be closely linked in stem cells which may be important for gene expression, and lineage programming in stem cell populations.

When transgenerational inheritance of phenotypic traits deviates from Mendelian inheritance patterns (e.g. when the phenotype such as obesity increases in the F3 generation rather than being diluted out), this is usually taken as evidence for epigenetic, rather than genetic effects. Although the data are still emerging, it may be the case that epimutations can increase the rate of nearby spontaneous genomic DNA mutations [73^{*}] and that changes in local DNA sequence can lead to epigenetic changes in the same region [74^{**}]. The analysis of the relative contributions of genetic and epigenetic changes to transgenerational inheritance is an important topic for future studies and the widespread adoption of deep-sequencing methods versus microarrays will enable these issues to be addressed.

Future directions

Recent decades have seen a large increase in the incidence of obesity in all age groups [1,2]. Despite increased public awareness of the problem, the obesity epidemic continues unabated and the medical community is finally beginning to consider the possibility that there may be more to obesity than 'calories in-calories out'. Work in our laboratory and in other labs around the world has identified obesogenic chemicals that can predispose exposed animals to accumulate more fat and these effects can be transmitted to their descendents (reviewed in [40,75^{*}]). The precise etiology of weight gain in exposed animals is uncertain — it could be that some eat more and exercise less — but this misses the point. The essential point of these studies is that chemical exposure, *per se*, has elicited weight gain in exposed animal independent of other factors. Since humans are exposed to the same obesogenic

chemicals that have been studied in animals (as well as others that could also be obesogenic), one might reasonably expect these exposures to contribute to increased adiposity over time.

The obesogen hypothesis currently poses more questions than it answers. There is much to be learned about molecular mechanisms underlying how chemical exposure produces the observed effects on adipogenesis, metabolism and obesity. Very little is known about windows of sensitivity within which obesogen exposure may be particularly damaging or whether some segments of certain populations may be more susceptible than others. Little is known about potential interactions between obesogen exposure and diet. Better coordination is needed between animal studies that can reveal mechanisms of action and human epidemiological studies that can identify whether the effects observed in animal studies are also observed in humans exposed to the same chemicals. Notwithstanding the difficulty of understanding how single obesogens act, we need to know what are the effects of chemical mixtures to which the human population is exposed. We also need a better understanding of which of the chemicals in commerce, including the 84,577 chemicals listed on the EPA Toxic Substances Control Act inventory [76], might cause adverse consequences, *in vivo*, including metabolic disturbances and obesity. It should also be noted that there is currently an epidemic of Type 2 diabetes that may involve an overlapping, but distinct set of EDCs than obesity [77].

Lastly, recent studies showing transgenerational inheritance of the effects of EDC exposure, including obesity, raises the stakes in the debate about the effects of EDC exposure. Our work has demonstrated that the exposure of environmentally relevant doses to TBT during *in utero* development is contributing to the development of metabolic alterations that are transmitted to subsequent generations. Although the usage of TBT nowadays is limited, a recent publication has demonstrated its presence in modern house dust [48]. This data suggests that we might not only inherited the phenotypes generated by TBT but we are also receiving direct exposure to it, making us carriers and transmitters of these phenotypes. The published work from Skinner *et al.*, suggests that TBT seems to be just one of an undetermined number of obesogens we are exposed to that can modulate our metabolism inducing fat storage and altering lipid homeostasis. Taken together, these findings give us some insights about the mechanisms by which the EDCs are contributing to the increase in body weight ratio in the population opening a new path to investigate the role of other EDCs in the obesity epidemic.

Conflict of interest statement

B.B. is a named inventor on U.S. patents 5,861,274, 6,200,802, 6,815,168, and 7,250,273 related to PPAR γ .

R.C.-G. declares that she has no actual or potential competing financial interests.

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