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Review

Transgenerational inheritance of prenatal obesogen exposure

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ABSTRACT

Obesity and metabolic syndrome diseases have exploded into an epidemic of global proportions. The generally accepted cause of obesity is overconsumption of calorie-dense food and diminished physical activity (the calories in–calories out model). However, emerging evidence demonstrates that environmental factors can predispose exposed individuals to gain weight, irrespective of diet and exercise. The environmental obesogen model proposes that chemical exposure during critical stages in development can influence subsequent adipogenesis, lipid balance and obesity. Obesogens are chemicals that inappropriately stimulate adipogenesis and fat storage. Numerous obesogens have been identified in recent years and some of these have been shown to act through the peroxisome proliferator activated receptor gamma, the master regulator of adipogenesis. Others act through as yet unidentified pathways. Notably, some of these obesogens elicit transgenerational effects on a variety of health endpoints, including obesity in offspring after exposure of pregnant F0 females. Thus, prenatal exposure to xenobiotic compounds can have lasting, potentially permanent effects on the offspring of exposed animals. Transgenerational effects of chemical exposure raise the stakes in the debate about whether and how endocrine disrupting chemicals should be regulated.

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1. Obesity is a growing problem

Obesity and related disorders are a public health epidemic, particularly in the U.S. Currently more than 35% of the U.S. population is clinically obese (body mass index – BMI > 30) and 68% are overweight (BMI > 25). These figures are more than double the worldwide

average and 10-fold higher than the rates in Japan and South Korea (Flegal et al., 2010; Ogden et al., 2014). Obesity and obesity-related disorders impose an estimated \$208 billion annual burden on the U.S. health care system (Cawley and Meyerhoefer, 2012), and childhood obesity can cost more than \$30,000 over the lifetime of an obese child (Finkelstein et al., 2014). Genetics (Herbert, 2008) and behavioral factors such as smoking (Power and Jefferis, 2002), stress (Garruti et al., 2008), a sedentary lifestyle (Rippe and Hess, 1998) and excessive consumption of food (Hill and Peters, 1998) are the typically cited causes of obesity. However, environmental factors such as sleep disruption (Watenpaugh, 2009), light pollution (Fonken et al., 2013), viral infection (Mittra and Clarke, 2010;

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van Ginneken et al., 2009), the composition of gut bacteria flora (Ley et al., 2005, 2006) and exposure to xenobiotic chemicals (Janesick and Blumberg, 2011) are emerging as significantly contributing factors to obesity. These environmental factors may interact with genetic or lifestyle factors to exacerbate the effects of diet and exercise, calling for a reassessment of the favored “calories in-calories out” model of obesity.

2. New approaches are needed

An alarming recent trend is the high rate of obesity in very young children, including infants (Koebnick et al., 2010; McCormick et al., 2010; Taveras et al., 2009). At least one study suggests that the rate of childhood obesity is reaching a plateau in some Western countries (Ogden et al., 2014), but this view is currently controversial. While one can argue that present-day children, adolescents and adults may be eating more and exercising less than in the past, this is unlikely to apply to infants. A typical infant eats until satiation and exercises very little; therefore, it is implausible that changes in caloric expenditure in infants have contributed to obesity at a young age. A more likely explanation is that the prenatal environment causes these overweight or obese infants to be born with more fat, to be predisposed to accumulate fat easily and/or that the early postnatal environment has changed significantly in recent years. In support of this hypothesis, a recent study showed that animals living in proximity to humans (pets – cats and dogs; laboratory animals – rats, mice, four species of primates; and feral rats) in industrialized societies exhibited pronounced increases in obesity over the past several decades (Klimentidis et al., 2011). While one could argue that our companion animals are pampered, overfed and under-exercised, the obese animal populations included laboratory animals living in strictly controlled environments, as well as feral animals living in cities (Klimentidis et al., 2011). The likelihood of 24 animal populations from eight different species all showing a positive trend in weight over the past few decades by chance was estimated at about 1 in 10 million (1.2×10^{-7}) – a vanishingly small possibility that this is a chance occurrence (Klimentidis et al., 2011). The most reasonable conclusion is that something has changed in the dwelling environment of these animals, making them obese in parallel with humans.

3. The obesogen hypothesis

In 2006, we proposed the existence of endocrine disrupting chemicals (EDCs) that could influence adipogenesis and cause obesity in animals and humans. This group of EDCs may be important, yet unsuspected players in the obesity epidemic. We define “obesogens” functionally as chemicals that promote obesity by increasing the number of fat cells and/or the storage of fat into existing adipocytes. Obesogens can also act indirectly to promote obesity by changing basal metabolic rate, by shifting energy balance to favor calorie storage, by promoting food storage via gut microbiota (Snedeker and Hay, 2012), and by altering hormonal control of appetite and satiety (Blumberg, 2011; Heindel, 2011; Janesick and Blumberg, 2011; La Merrill and Birnbaum, 2011; Newbold, 2011). Several obesogenic chemicals have been identified in recent years, underscoring the relevance of this new model. Estrogenic EDCs such as diethylstilbestrol (DES) (Newbold et al., 2009), bisphenol A (BPA) (Rubin, 2011; Rubin et al., 2001) and DDT (Skinner et al., 2013), organotins such as tributyltin (TBT) (Chamorro-Garcia et al., 2013; Grun et al., 2006), perfluorooctanoates (Hines et al., 2009) and phthalates (Hao et al., 2012, 2013; Manikkam et al., 2013) are obesogenic in animals. Urinary phthalate levels were correlated with increased waist diameter (Hatch et al., 2008; Stahlhut et al., 2007) and high levels of several persistent organic pollutants (e.g., DDE, HCB, polybrominated diphenylethers) were linked with obesity in humans (Tang-Peronard et al., 2011). Because this topic has been exten-

sively reviewed in recent years, this review will focus on transgenerational effects of obesogenic chemicals and potential mechanisms through which they might act.

4. How do obesogens act?

The only obesogens with an unambiguously demonstrated pathway of action are TBT, and by implication triphenyltin (TPT). TPT is widely used in agriculture and TBT in industry. Human exposure to organotins occurs through dietary sources (seafood and shellfish), from organotin use 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 (Golub and Doherty, 2004; Grun and Blumberg, 2006; Okoro et al., 2011). Organotins have also been found in appreciable levels in house dust, suggesting that exposure is widespread (Kannan et al., 2010). TBT and TPT are high-affinity ligands for two nuclear receptors critical for adipocyte development: the 9-cis retinoic acid receptor (RXR) and peroxisome proliferator activated receptor gamma (PPAR γ), in vitro and in vivo (Grun et al., 2006; Kanayama et al., 2005). TBT promotes adipogenesis in murine 3T3-L1 pre-adipocytes (Grun et al., 2006; Kanayama et al., 2005) and in human and mouse multipotent mesenchymal stromal cells (MSCs, a.k.a. mesenchymal stem cells) via a PPAR γ -dependent pathway (Kirchner et al., 2010; Li et al., 2011). In utero TBT exposure leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and increased adipose depot mass in adult mice (Chamorro-Garcia et al., 2013; Grun et al., 2006). Exposure of adolescent or adult mice to TBT causes increased fat depot size, accumulation of lipids in the liver and insulin resistance (Zuo et al., 2011, 2014). Placental TBT levels are positively correlated with weight gain in human male infants at 3 months of age (Rantakokko et al., 2014). Thus, although more data are needed, TBT exposure is associated with weight gain both in animals and in humans.

5. Adipogenesis in a nutshell

Adipogenesis is a differentiation event in the mesodermal lineage wherein MSCs and their more lineage-restricted derivatives give rise to adipocytes, both during development and to maintain fat cell number in adulthood (Cristancho and Lazar, 2011; Rosen and MacDougald, 2006). MSCs are thought to reside largely in the perivascular niche of most organs (Crisan et al., 2008) and some authors have suggested that they are identical to pericytes that surround most blood vessels (Crisan et al., 2008, 2009). MSC/pericytes can give rise to a variety of cell types in culture (adipose, bone, cartilage, muscle, etc.) upon stimulation with specific differentiation cocktails (Bianco, 2011). It is currently unclear whether MSC/pericytes located in different tissues normally have a restricted lineage differentiation potential in vivo, or whether they have the same broad lineage potential, in vivo, as they appear to have, in vitro (Bianco, 2011).

While many studies have demonstrated how cells already committed to the adipocyte lineage differentiate into mature adipocytes (Tontonoz and Spiegelman, 2008), we know much less about the mechanisms and intermediates through which MSCs become committed to the adipocyte lineage (Rosen and Spiegelman, 2014) and how this process might be influenced by EDCs. Bone morphogenic proteins (BMPs), Wnt, and PI3K/Akt signaling are important parts of the adipocyte commitment pathway, probably mediated by the expression of genes such as Zfp423 (Gupta et al., 2010), Zfp521 (Kang et al., 2012), TCF7-like 1 (Cristancho and Lazar, 2011), and S6K1 (Carnevali et al., 2010). MSCs give rise to both adipocytes and osteoblasts; the commitment to one or the other lineage is mutually exclusive (Shockley et al., 2007). Expression of PPAR γ commits cells to the adipogenic lineage whereas Wnt signaling inhibits PPAR γ

expression and diverts MSCs toward the osteogenic lineage (Cristancho and Lazar, 2011; Takada et al., 2009). Repression of non-canonical Wnt-5a (Takada et al., 2007) and canonical Wnt-3a/10b (Kang et al., 2007; Kawai et al., 2007; Ross et al., 2000) signaling together with active BMP/TGF- β and PI3K/Akt signaling (Carnevali et al., 2010; Kang et al., 2009; Zamani and Brown, 2011) is required for MSCs to proceed toward the adipogenic and away from the osteogenic lineage. Non-coding RNAs are also emerging contributors to promote the differentiation of stromal cells to adipocytes (Sun et al., 2013). Therefore, studying the commitment of MSCs to different lineages provides unique opportunities to evaluate the effects of early life exposure to EDCs on the development of fat depots and obesity.

6. EDCs and reprogramming of MSC fate

The involvement of multiple signaling pathways to switch MSCs between adipogenic and osteogenic fates offers many possibilities for disruption by EDCs; however, only a few studies have directly tested how EDCs might influence MSC fate. The pesticides chlorpyrifos and carbofuran inhibited the ability of MSCs to differentiate into bone (Hoogduijn et al., 2006) but the potential of these cells to differentiate into fat was not tested. We found that treatment with the environmental obesogen, TBT, or the pharmaceutical obesogen, rosiglitazone (ROSI) led to adipogenic differentiation of 3T3-L1 preadipocytes *in vitro* and prenatal exposure of pregnant mice to these chemicals led to increased fat deposition at birth (Grun et al., 2006). The widely used fungicide, triflumizole, also promoted adipocytic differentiation and gene expression in MSCs and 3T3-L1 cells; this effect disappeared when cells were treated with triflumizole in the presence of a PPAR γ antagonist (Li et al., 2012). Prenatal TBT or ROSI exposure induced cultured naive MSCs to differentiate into adipocytes in a PPAR γ -dependent manner; prenatal exposure of laboratory animals to TBT or ROSI significantly enhanced commitment of MSCs isolated from bone marrow or adipose tissue to the adipogenic lineage at the expense of the osteogenic lineage (Kirchner et al., 2010). This reprogramming of lineage commitment observed in cell culture is reflected *in vivo* with larger adipose depots and adipocytes as well as increased expression of adipogenic markers and decreased expression of bone markers in MSCs on a normal chow diet containing only 4.5% of calories from fat (Chamorro-Garcia et al., 2013).

Our recent study demonstrated that the effects of prenatal TBT exposure on fat depot size and MSC reprogramming persist through at least the F3 generation after exposure of pregnant F0 animals (Chamorro-Garcia et al., 2013). This observation suggests that prenatal TBT exposure has caused heritable alterations in the germ cell genome of the directly exposed F1 fetuses that predisposes the MSC compartment toward the adipocyte lineage and away from the osteogenic lineage. This may be an example of a maternal programming event that permanently alters stem cell fate leading to a reproducible adult phenotype. Nothing is currently known about how obesogen exposure causes heritable changes in the genome that alter MSC fate. Transgenerational effects in F3 and beyond are distinguished from multigenerational effects in F1 and F2 offspring. F1 and F2 animals are directly exposed to the chemicals *in utero* and phenotypes may result from this exposure. F3 animals are never exposed to chemical and any effects seen in F3 and later generations must result from epigenetic (or genetic) alterations. In addition to our studies, Skinner and colleagues have also shown transgenerational effects of several environmental chemicals on obesity in rats. These include plastic components such as BPA, diethylhexyl phthalate and dibutyl phthalate (Manikkam et al., 2013), a mixed hydrocarbon mixture (jet fuel JP-8) (Tracey et al., 2013) and perhaps most significantly, the once widely used pesticide, DDT (Skinner et al., 2013). Whether DDT acts through estrogen recep-

tors, and DDE, its primary metabolite acts via the androgen receptor remains to be demonstrated; little is known about the mechanisms through which the other chemicals act. Transgenerationally inherited sperm epimutations that could be involved in the etiology of obesity and other disease outcomes have been identified (Guerrero-Bosagna et al., 2014). This is an active research area in multiple laboratories and much progress is expected in the near future.

7. Epigenetic vs. genetic changes

Epigenetic modifications can affect gene expression during development, cellular differentiation, and in response to environmental stimuli. DNA methylation was proposed as a key mechanism mediating adult diseases with developmental origins (Ho and Tang, 2007). Distinct patterns of CpG methylation are critical for gene silencing and aberrant patterns of DNA methylation influence many aspects of disease processes (Zhang and Ho, 2011). Changes in DNA methylation may be a key mechanism for the transgenerational effects of environmental exposure to chemicals as well as nutritional deficits (Zhang and Ho, 2011). Recently, alterations in DNA methylation at metastable alleles were shown to differ in human infants that were conceived during harvest (associated with increases in the ingestion of methyl-donors) or during famine (Dominguez-Salas et al., 2014). Biomarkers of one-carbon substrates in maternal blood during periconception were linked to alterations in DNA methylation in the child, thus implicating a mechanism for potential life-long or transgenerational effects due to maternal nutrition in humans (Dominguez-Salas et al., 2014). Given that exposures of laboratory rodents to environmental chemicals can also change metastable alleles (e.g., *agouti*) (Bernal and Jirtle, 2010), permanent epigenotoxic effects may also be occurring in EDC-exposed human fetuses.

In addition to DNA methylation (5-mC), 5' hydroxymethylation at cytosines (5-hmC) is dynamically regulated in stem cells and during their differentiation (Ficz et al., 2011). 5 hmC are prevalent near transcription start sites in the promoters of genes expressed in stem cells, playing important roles in regulating expression of critical genes for development and differentiation (Szulwach et al., 2011; Tahiliani et al., 2009; Veron and Peters, 2011; Wu et al., 2011). Other studies showed that 5-hmC is involved in regulating several pluripotency factors in stem cells (Ficz et al., 2011; Ito et al., 2010; Wu et al., 2011). Recent studies established that 5-hmC is an important intermediate metabolite generated during the active DNA demethylation process, in which 5-mCs are oxidized to 5-hmCs by the Tet family methylcytosine dioxygenases and then 5-hmCs (as well as their metabolites 5'-formyl and 5'-carboxyl cytosines and 5-hydroxymethyluracils) are removed from the DNA by the base excision repair mechanism (Guilbert and Weber, 2013; Serandour et al., 2012). Interestingly, during the adipocytic differentiation of 3T3-L1 preadipocytes, PPAR γ recruits Tet enzymes to its binding sites in adipogenic gene promoters causing local demethylation (Fujiki et al., 2013), decreased 5-mC and increased 5-hmC (Oger et al., 2014). Thus, there may be a close correlation between these two methylation processes in stem cells including MSCs and possible effects of obesogens on formation and metabolism of 5-hmC should also be examined in future studies.

EDC-initiated phenotypes may be transmitted over generations via epigenetic alterations such as DNA methylation or histone tail modifications. However, possible roles of genetic changes directly affecting DNA base sequences cannot be excluded. While deviation from a Mendelian inheritance pattern of the transgenerational traits is often taken as evidence for an epigenetic cause of transgenerational effects, such data do not exclude the involvement of genetic alterations in the EDC-initiated phenotypes. An emerging concept is that some epigenetic changes might

be harmful only in the presence of concomitant nucleotide base mutations in functionally relevant genes, whereas they remain silent in the absence of these mutations. This hypothesis proposes that some epimutations may require additional, and probably specific, nucleotide base mutations to manifest their phenotypes. For example, enhanced penetrance of uterine leiomyoma was observed after developmental exposure to diethylstilbestrol in rats carrying loss-of-function mutations in the *Tsc2* tumor suppressor gene, but not in wild type animals (Cook et al., 2005; Greathouse et al., 2008). Recent data show that epigenetic changes increase the rate of spontaneous genomic DNA mutations nearby (Schuster-Bockler and Lehner, 2012) and that nucleotide base mutations in genomic DNA are associated with epigenetic changes in the same region (You and Jones, 2012). These data indicate that the search for molecular mechanisms underlying EDC-initiated transgenerational phenotypes should be broadened to include the possibility that epigenetic and genetic mechanisms may interact or cooperate. Accordingly, techniques that can detect both epigenetic marks and genomic DNA mutations (e.g., deep sequencing and pyrosequencing) will be very advantageous for fully understanding the etiology of epigenetic phenotypes observed.

8. Significance and future prospects

As the obesity epidemic continues in the U.S. and expands throughout the world, there is an urgent need to understand the mechanisms underlying the predisposition to obesity and related disorders. While evidence implicating environmental influences continues to mount, studies investigating the importance of environmental pollutants as factors in obesity are only beginning, and the mechanisms through which environmental obesogens contribute to the generation of obesity remain largely unknown. The obesogen hypothesis opened a new area of research into obesity by connecting endocrine disruptor research with developmental origins of disease. The obesogen hypothesis is still relatively new and there is a dearth of research in this area, particularly with regard to mechanisms, developmental time windows, and interactions with diet. Our published work showed that PPARγ activators such as TBT and triflumizole modulate critical steps of adipogenesis in vitro and in vivo through RXR/PPARγ and that prenatal exposure to these obesogens predisposes MSCs to become adipocytes by epigenetic imprinting into the memory of the MSC compartment (Kirchner et al., 2010; Li et al., 2012). Our recent studies showed that the effects of prenatal TBT exposure are transgenerational; exposure of pregnant F0 animals to environmentally-relevant levels of TBT produced obesogenic effects that persisted through at least the F3 generation (Chamorro-Garcia et al., 2013). Skinner and colleagues showed that a variety of other EDCs and environmental contaminants exert transgenerational effects on numerous health endpoints, including obesity (Manikkam et al., 2013; Skinner et al., 2013; Tracey et al., 2013). It will be important to investigate the magnitude of the effects of such environmental obesogenic pollutants on the obesity epidemic and the extent to which regulatory actions on such chemicals would improve the public health. It will be important to uncover the mechanisms through which prenatal obesogen exposure can be engraved in the stem cell compartment memory and more generally how EDCs can elicit transgenerational effects on stem cell fate. Unraveling the complex modulation of developmental pathways controlling the prenatal programming of MSC fate will make important contributions to understanding the development of obesity and how obesogens affect this process. If these observations in animals are applicable to humans (as is highly likely), it means that our dietary habits and chemical exposure may permanently predispose our descendants toward an adverse metabolic profile which could lead to obesity and related disorders.

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