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Transgenerational Inheritance of Increased Fat Depot Size, Stem Cell Reprogramming, and Hepatic Steatosis Elicited by Prenatal Obesogen Tributyltin in Mice

Raquel Chamorro-García¹, Margaret Sahu¹, Rachele J. Abbey¹, Jhyme Laude¹, Nhieu Pham¹, and Bruce Blumberg^{1,2}

¹Department of Developmental and Cell Biology, University of California, Irvine, California, USA

²Department of Pharmaceutical Sciences, University of California, Irvine, California, USA

Corresponding Author: Bruce Blumberg, U.C. Irvine, 2011 BioSci 3, Irvine, CA 92697-2300, phone 949-824-8573, fax 949-824-4709, Email: blumberg@uci.edu.

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Abbreviations

ACOX, acyl Coenzyme A oxidase; ADIPOQ, Adiponectin; ALP, Alkaline Phosphatase; BAT, brown adipose tissue; DMSO, dimethylsulfoxide; EDC, Endocrine Disrupting Chemical; FABP4, Fatty Acid Binding Protein 4; FASN, fatty acid synthase; FATP, fatty acid transport protein; Fsp27, fat-specific protein of 27 kDa; GyK, glycerol kinase; LPL, Lipoprotein Lipase; α -MEM, Minimal Essential Medium without nucleosides; MSC, Multipotent Mesenchymal Stromal Stem Cell; NAFLD, non-alcoholic fatty liver disease; NOAEL, no observable adverse effect level; PPAR, Peroxisome Proliferator Activated Receptor; Pref-1, Adipocyte Differentiation-associated Protein-1/Preadipocyte Factor-1; QPCR, quantitative real-time reverse transcriptase polymerase chain reaction; ROSI, Rosiglitazone; RXR, Retinoid 'X' Receptor; Runx2, Runt Related Transcription Factor 2; SREBP1c, sterol response element binding protein 1c; TBT, tributyltin; zfp423, zinc finger protein 423

Abstract

Background: We previously showed that exposure to tributyltin (TBT) modulates critical steps of adipogenesis through RXR/PPAR γ and that prenatal TBT exposure predisposes multipotent mesenchymal stem cells (MSCs) to become adipocytes by epigenetic imprinting into the memory of the MSC compartment.

Objectives: We tested whether the effects of prenatal TBT exposure were heritable in F2 and F3 generations.

Methods: We exposed C57BL/6J female mice to DMSO vehicle, the pharmaceutical obesogen rosiglitazone (ROSI) or three doses of TBT (5.42 nM, 54.2 nM and 542 nM) throughout pregnancy via the drinking water. F1 offspring were bred to yield F2 and F2 bred to produce F3 generations. F1 animals were exposed *in utero*, F2 were potentially exposed as germ cells in the F1 whereas F3 animals were never exposed to the chemicals. We analyzed the effects of these treatments on fat depot weights, adipocyte number, adipocyte size, MSC programming, hepatic lipid accumulation and hepatic gene expression in all three generations.

Discussion: Prenatal TBT exposure increased most white adipose tissue (WAT) depot weights, adipocyte size, adipocyte number and reprogrammed MSCs toward the adipocyte lineage at the expense of bone in all three generations. Prenatal TBT exposure led to hepatic lipid accumulation and up-regulated hepatic expression of genes involved in lipid storage/transport, lipogenesis and lipolysis in all 3 subsequent generations.

Conclusions: Prenatal TBT exposure produced transgenerational effects on fat depots and induced a phenotype resembling nonalcoholic fatty liver disease through at least the F3 generation. These results show that early life obesogen exposure can have lasting effects.

Introduction

Emerging evidence supports the idea that prenatal and early postnatal events such as maternal nutrition, drug, and chemical exposure are manifested in health consequences later in life (reviewed in Barker 2007; Janesick and Blumberg 2011). Obesity is a burgeoning epidemic in the developed world over the past 30 years (Flegal et al. 2010). Obesity, together with other metabolic risk factors such as insulin resistance, hypertension and non-alcoholic fatty liver disease (NAFLD), is strongly correlated with the development of cardiovascular disease and diabetes and the total cost of obesity related disorders has been estimated at \$208 billion (in 2008 dollars), or ~21% of health care costs in the United States alone (Cawley and Meyerhoefer 2012). It is widely believed that an increase in fat mass occurs as the primary result of impaired balance between energy intake and energy expenditure; however, additional factors are important contributors to obesity. These include smoking, stress, sedentary lifestyle, excessive consumption of alcohol and genetics (Garruti et al. 2008; Herbert 2008; Hill and Peters 1998; Power and Jefferis 2002; Rippe and Hess 1998). Epidemiological studies in human populations have demonstrated a correlation between parental obesity and the development of obesity during their children's childhood (Danielzik et al. 2002; Power et al. 1997). Although little is known about the underlying mechanisms, one recent study demonstrated a correlation between the epigenetic state of DNA from the umbilical cord of children and adiposity later in their childhood (Godfrey et al. 2011). There are currently no studies that have examined whether such epigenetic modifications can be transmitted to subsequent generations in humans.

Recent work from our laboratory and from other laboratories supports a role for environmental factors in the development of obesity; these factors include exposure, especially during critical developmental windows, to endocrine disrupting chemicals (EDCs) (reviewed in

Heindel 2011; Janesick and Blumberg 2011; La Merrill and Birnbaum 2011; Newbold et al. 2009; Tang-Peronard et al. 2011). “Obesogens” are chemicals that promote obesity directly by increasing adipocyte size and/or number, or indirectly by altering metabolic setpoints or interfering with the regulation of appetite and satiety. Our environmental obesogen hypothesis proposes that a subset of EDCs could promote the development of obesity. Although initially controversial, the obesogen hypothesis has gained momentum in recent years with the identification of obesogenic chemicals that promote adipogenesis and obesity in animals and humans (reviewed in Janesick and Blumberg 2011). Our published work identified tributyltin (TBT) as an environmental obesogen. Prenatal TBT exposure increased adipose depot size in mice via activation of the peroxisome proliferator activated receptor gamma (PPAR γ) (Grun et al. 2006; Li et al. 2011) and reprogrammed the fate of multipotent mesenchymal stromal stem cells (MSCs) to favor the adipogenic lineage at the expense of the osteogenic lineage, *in vitro* and *in vivo* (Kirchner et al. 2010).

There is a growing body of evidence showing that developmental exposure to EDCs leads to adverse health outcomes later in life (reviewed in Diamanti-Kandarakis et al. 2009). EDCs can act directly on nuclear hormone receptors, on a variety of transcriptional cofactors, on enzymatic pathways involved in hormone biosynthesis or metabolism and on neuroendocrine signaling pathways. Many effects of EDC exposure are manifested as disturbances of endocrine or reproductive systems (Diamanti-Kandarakis et al. 2009) and recent evidence suggests that some of these phenotypes can be transmitted to subsequent generations (Anway et al. 2005; Anway et al. 2008; Blatt et al. 2003; Goncalves et al. 2010; Nilsson et al. 2012; Wolstenholme et al. 2012a; Zambrano et al. 2005). Until now, it was unknown whether the obesity-related effects of obesogen exposure were heritable in a multi- or transgenerational manner.

In this work, we exposed pregnant F0 mice to three different concentrations of TBT (5.42 nM, 54.2 nM and 542 nM) in the drinking water that deliver, respectively, ~50 fold lower, ~5 fold lower and ~2 fold higher doses compared with the established no observable adverse effect level (NOAEL) of 25 µg/kg/day (Vos et al. 1990). F1 animals were exposed directly during the *in utero* development while F2 animals were potentially exposed as germ cells of the F1. Effects noted in F1 and F2 generations are termed multi-generational (Skinner 2008). F3 animals are the first generation that received no exposure to TBT at any time and phenotypes observed in F3 animals are considered to be transgenerational and permanent (Anway and Skinner 2006; Jirtle and Skinner 2007).

Here we show that exposure of pregnant F0 mice to TBT led to effects on exposed F1 animals as well as their F2 and F3 descendants. Offspring of mice exposed prenatally to TBT and their descendants exhibited increased adipose depot weight, larger adipocyte size, increased adipocyte number and biased cell fate in the MSC compartment to favor the adipocyte lineage at the expense of the bone lineage, despite eating a normal diet compared with control animals. TBT exposed animals and their descendants developed fatty livers and exhibited altered hepatic gene expression suggestive of NAFLD in all three generations. Our results demonstrate that the effects of prenatal TBT exposure are permanent and transgenerational, suggesting an increased risk for future generations to develop obesity and related disorders such as NAFLD. These results have important implications for the ongoing debate about developing appropriate policies to minimize the negative effects of EDC exposure.

Methods

Animal husbandry

Male and female C57BL/6J mice (8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in micro-isolator cages in a temperature-controlled room (22–24° C) with a 12-h light, 12-h dark cycle and provided water and food (standard low-fat diet for rodents RMH 2500; Purina Mills, Richmond, IN) *ad libitum*. Animals were treated humanely and with regard for alleviation of suffering. All procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of the University of California Irvine.

Fetal exposure to TBT

Female C57BL/6J mice were exposed, via drinking water, to three different concentrations of TBT (5.42 nM, 54.2 nM, 542 nM), 500 nM rosiglitazone (ROSI) or DMSO vehicle (all of which were diluted in 0.5% carboxymethyl cellulose in drinking water to maximize solubility) (6 females/treatment) during 7 days before mating. These concentrations of TBT represent ~50 fold lower, ~5 fold lower or ~2 fold higher doses than the NOAEL. Plug detection was defined as embryonic day 0.5. Chemical treatment was provided to the females through the end of pregnancy so the F1 animals were exposed to the chemicals during the *in utero* development while the F2 animals was exposed as the germ cells of the F1. F3 animals were not exposed to the chemicals. F1 and F2 animals from different litters of each treatment group were mated to each other avoiding sibling inbreeding (6 females and 6 males per group). We only analyzed those litters with a number of pups between 5 and 7, so the final number of

litters per group analyzed was 4-5 (Supplemental Materials Table S1). There were no statistically significant differences in the number of pups per litter among the different groups and we considered both male and female offspring in our analysis. Mice were euthanized at 8 weeks of age (between 8-16 animals per group; Supplemental Materials Table S2) by cervical dislocation.

Tissue isolation and histological sections

Mice were dissected to isolate and weigh epididymal/ovarian, peri-renal and interscapular white and brown adipose tissues. These tissues were divided in two groups: 1) 3.7% formaldehyde fixation and hematoxylin and eosin (H&E) staining and 2) DNA isolation. The samples were embedded in paraffin, sectioned, and prepared for H&E staining following standard protocols in the core facility of Department of Pathology and Laboratory Medicine at the University of California, Irvine. Bright field pictures were acquired on Zeiss Axiovert 40 CFL microscope (40x magnification). Adipocyte size was measured with Adobe Photoshop CS5. Adipocyte areas were selected, measured and the area expressed as the number of pixels per selected area. Measurements for 100 cells from each animal were averaged. Brown adipose tissue lipid content is shown as the percentage of the area covered by lipid vesicles, measured by using ImageJ (Schneider et al. 2012).

For DNA isolation, 1 mL of RLT buffer (Qiagen) was added to 20 µg of each adipose tissue sample. Tissues were homogenized with a Pellet Pestle Motor (Kontes). DNA quantitation was performed as previously described with Quanti-it™ PicoGreen® dsDNA Reagent (Invitrogen) (Kanno et al. 2006).

The left lobe of the liver of each animal was isolated, fixed in 3.7 % formaldehyde and kept at 4°C overnight. They were washed with 1 x PBS during 24h and kept in 30% sucrose.

Tissues were embedded in OCT, flash frozen and stored at -20°C for subsequent sectioning (10 µm) in a cryostat. Sections were rinsed with distilled water, then 60% isopropanol in distilled water, then stained for 20 minutes with Oil Red O (4 g/L, 60% isopropanol). The slides were washed with 60% isopropanol to avoid Oil Red O precipitation and subsequently washed with 1 x PBS for 5 minutes, then samples were counter-stained with hematoxylin. At least 5 livers per group were analyzed and a representative picture was selected for the final figure. Differential Interference Contrast (DIC) pictures were acquired on Zeiss Axioplan II microscope (40x magnification).

Mesenchymal stem cells isolation

Bone marrow mesenchymal stem cells (MSCs) were isolated from femurs and tibia by flushing them with media and expanding them in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf bovine serum, 2mM sodium pyruvate and 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in subconfluent culture as previously described (Chamorro-Garcia et al. 2012).

Quantitative real time RT-PCR (QPCR)

Total RNA was extracted from MSCs and livers using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD). Complementary DNA was generated from 2µg DNase-treated RNA using Transcriptor Reverse Transcriptase (Roche, Nutley, NJ) following the manufacturer recommended protocol. Real-time PCR was performed in the DNA Engine Opticon Thermal Cycler (MJ Research/Bio-Rad Laboratories, Hercules, CA). QPCR was performed with FastStart SYBR Green QPCR Master Mix (Roche, Nutley, NJ) and 100 nM of primers (listing in

Supplemental Materials Table S3). Primers were chosen using Primer3 software (Rozen and Skaletsky 2000) and verified to produce a single peak by gel electrophoresis and melting curve analysis. Relative quantification of the target gene transcript in comparison with β -actin (housekeeping gene) expression levels in the same sample followed the $\Delta\Delta^{Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Data are presented as means \pm SEM. One way ANOVA with Dunnett's posthoc test was used to determine the significance of the differences in measured outcomes among fat depot weight, adipocyte size, adipocyte number and body weights from groups with different treatments. $P < 0.05$ was considered statistically significant. There was no statistically significant variation in litter representation among DMSO, ROSI, or TBT groups or across generations (DMSO vs. TBT $P = 0.2743, 0.3965,$ and 0.5174 for F1, F2 and F3 respectively); therefore, tissues from individual animals were analyzed rather than pooling data from litters. QPCR analysis used unpaired t-tests. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used to perform the statistical analysis.

Results

TBT exposure elicits transgenerational effects on adipose depot weight, adipocyte size and adipocyte number

Our published studies demonstrated epigenetic changes in PPAR γ target gene expression and stable changes in MSC programming (Kirchner et al. 2010). Therefore, we asked

whether the effects of prenatal TBT exposure could be passed on to subsequent generations. Female C57BL/6J mice were treated prior to conception and then throughout pregnancy with vehicle (DMSO), the pharmaceutical obesogen ROSI or the environmental obesogen TBT. The F1 offspring of these mice (from different litters) were bred to produce F2 and these were bred to yield F3 animals. F1 animals were directly exposed *in utero* to TBT whereas F2 animals were potentially exposed as germ cells within the embryonic F1 animals. The F3 generation was never exposed to the chemicals and any changes observed in F3 are considered to be transgenerational and permanent (Anway and Skinner 2006; Jirtle and Skinner 2007).

We analyzed overall body weight, fat depot weight, MSC gene expression, hepatic lipid accumulation, and hepatic gene expression in 8 week old mice (the maximum age from which we could reliably prepare viable MSCs) considering both male and female offspring. To ascertain whether changes in fat depot weight were due to hypertrophy, hyperplasia or both, we measured adipocyte size and the depot DNA content per mg of tissue to determine adipocyte number in all white (WAT) and brown (BAT) adipose tissues isolated. Two visceral depots were sampled, the epididymal/ovarian WAT and the peri-renal WAT. The subcutaneous depot analyzed was the interscapular WAT and we dissected brown adipose tissue (BAT) away from the interscapular WAT for separate analysis.

We observed striking increases in WAT depots of treated animals. TBT treatment led to significant increases in the weights of two out of three WAT depots in F1 males and substantial increases in adipocyte size and number (Figure 1; See Supplementary Materials Figure S1 for representative sections), particularly in visceral WAT depots. Intriguingly, the increased number and size of adipocytes in F1 males was not completely reflected in the overall depot weights, which showed modest increases, suggesting that there may be a difference in the density of lipids

stored in the WAT of TBT treated animals (Figure 1). The effects on fat depot weights in F2 males were more marked than in F1, with all visceral WAT depots showing substantial and significant increases in animals derived from parents exposed to all doses of TBT exposed parents whereas the subcutaneous WAT depot only showed effects at the two highest doses of TBT (Figure 1A). In contrast to TBT, there was little change in WAT depot weights in response to ROSI treatment. The epididymal WAT depot weight increased in ROSI-treated F1 males whereas adipocyte size was increased but the number of cells did not change; other WAT depots showed no increases in weight and no change to decreased adipocyte size and number. Adipocyte size (Figure 1B) and number (Figure 1C) was increased in all F2 WAT depots from TBT treatments. F3 males from the TBT treatment group also showed large and significant increases in the weights of all WAT depots as well as in adipocyte size and number (Figure 1) (although not in all depots at every dose), demonstrating that the effects of prenatal TBT exposure were fully transgenerational in males.

Females showed more modest changes in fat depot size, although most doses of TBT lead to significant increases in WAT depot weight and adipocyte size in F1 and F2 animals (Supplementary Materials, Figure S2A-B). The number of cells was decreased in the ovarian WAT depot from F1 and F3 animals, but increased in the peri-renal and interscapular depots of F1-F3 animals (although not at every TBT dose) (Supplementary Materials Figure S2C). The peri-renal depot increased in size in F3 females, but there were no increases in ovarian and interscapular WAT in F3 animals at 8 weeks of age. However, adipocyte size remained elevated in the visceral WAT but was slightly decreased or unchanged in interscapular WAT (Supplementary Materials Figure S2B). Adipocyte number was decreased (ovarian) to slightly

increased (peri-renal, interscapular) in the WAT of F3 females (Supplementary Materials Figure S2C).

In contrast to the strong transgenerational effects on WAT depot size, effects on body weight in both males and females were less pronounced in these 8-week old animals. F1 animals showed very modest effects with ROSI leading to a slight increase in males and the highest dose of TBT (542 nM) leading to a slight (but not statistically significant) decrease in females (Supplementary Materials Figure S3). Animals in the TBT treatment group exhibited significant differences in body weight in F2 males but not females. F3 males showed a slight decrease at the lowest dose of TBT and a slight increase in females at 54.2 and 542 nM. Overall, we conclude that the effects on body weight at 8 weeks are modest, except for F2 males, probably because these animals are young and were maintained on normal chow.

Effects on depot size, adipocyte size and the number of cells in the BAT were modest in both males and females. We observed a trend toward decreased BAT weight in all 3 generations of males and females that only rarely reached statistical significance in TBT animals (Figure 1A, Supplementary Materials Figure S2A). There was an accompanying increase in the area covered by lipid vesicles in F1-F3 males and F1 females (Figure 1B, Supplementary Materials Figure S2B)(although not at every dose of TBT). Histological analysis showed that the BAT in these animals had large vesicles, unlike DMSO or ROSI-treated animals (Supplementary Materials Figure S1). Males trended toward reduced numbers of brown adipocytes in all 3 generations (only significant in F2), whereas females only showed strong changes in the number of cells in F2 animals (Figure 1C, Supplementary Materials Figure S2C).

TBT exposure causes a transgenerational reprogramming of MSCs to favor the adipocyte lineage.

MSCs are found in many tissues in the body, including bone marrow and adipose tissues. Depending on the stimuli they receive from surrounding cells or in culture, MSCs can differentiate into a variety of specialized cells such as osteoblasts, adipocytes, chondrocytes or myocytes, among others (reviewed in Pittenger et al. 1999). We previously showed that MSCs from mice exposed *in utero* to TBT were predisposed to differentiate into adipocytes at the expense of osteoblasts (Kirchner et al. 2010). To ascertain whether this effect is transmitted to the offspring of exposed animals, we analyzed the mRNA levels of a panel of adipogenic and osteogenic markers in MSCs obtained from the bone marrow of F1, F2 and F3 mice.

Early markers of enhanced adipogenic fate tested included zinc finger protein 423 (Zfp423), a transcriptional regulator of preadipocyte determination thought to function by inducing PPAR γ expression (Gupta et al. 2010), PPAR γ , considered to be the master regulator of adipogenesis (Tontonoz and Spiegelman 2008), and fatty acid binding protein 4 (Fabp4), a preadipocyte marker whose gene is a direct PPAR γ target (Burriss et al. 1999; Ibrahimi et al. 1994). Preadipocyte factor-1 (Pref-1) is an inhibitor of adipocyte differentiation (Sul et al. 2000) that we previously showed was down-regulated by obesogen exposure in MSCs (Kirchner et al. 2010; Li et al. 2011; Li et al. 2012). We also analyzed LPL, an adipogenic marker that functions to hydrolyze triglyceride bonds in lipoproteins, generating free fatty acids that can be taken up by cells and stored as triacylglycerols after esterification. LPL is also expressed in MSCs committed to the adipogenic lineage (Braun and Severson 1992).

QPCR analysis of bone marrow derived MSCs revealed sharply increased expression of adipogenic markers and decreased expression of Pref-1 in males from all 3 generations, with the

most dramatic effects in the F3 generation (Figure 2A). Data from female-derived MSCs largely followed the same trend but the changes were less pronounced in F2 and somewhat variable depending on the TBT dose in F3 (Figure S4A). Similar to the effects seen in males, the decrease in Pref-1 expression was most pronounced in F3 (Supplementary Materials Figure S4A).

To address potential changes in osteogenic capacity, we analyzed the early osteogenic markers bone specific alkaline phosphatase (ALP) and runt-related protein 2 (Runx2). ALP and Runx2 expression was sharply decreased in F1 and F3 males but unchanged in F2 (Figure 2B). Female MSCs showed strongly decreased ALP and Runx2 expression in F1 and F3 generations with a slight decrease in F2 (Supplementary Materials Figure S4B). F2 males did not show a significant change in osteogenic commitment, despite the increased adipogenic commitment (Figure 2B) which is not consistent with the prevailing view that commitment to fat or bone lineages is mutually exclusive (Beresford et al. 1992; Gimble et al. 2006; Takada et al. 2009). The changes observed in F3 males and females supports the contention that prenatal TBT exposure has caused a transgenerational reprogramming of MSC fate to favor the adipogenic, at the expense of the osteogenic lineage.

Prenatal TBT exposure induces fatty livers

Non-alcoholic fatty liver disease (NAFLD) is a risk factor associated with cardiovascular diseases and type 2 diabetes (reviewed in Perseghin 2011). Increased visceral adipose tissue leads to increased levels of circulating free fatty acids, which are taken up by the liver leading to triglyceride synthesis and accumulation (Gastaldelli et al. 2007). We observed that livers from F1 TBT-treated animals were noticeably whiter than controls (Supplementary Materials Figure S5); therefore, we analyzed livers from all generations for lipid accumulation and the expression

of genes involved in lipogenesis, lipolysis and lipid droplet storage. We found that F1 males and females exhibited a pronounced increase in lipid accumulation at all TBT concentrations (Figure 3). TBT has been shown to cause hepatic steatosis in adult male mice exposed at puberty (Zuo et al. 2011) but this is the first report that prenatal TBT treatment can cause the same phenotype. This increased lipid accumulation was also noted in F2 and F3 generations, but was less pronounced histologically (Figure 3). ROSI increased hepatic lipid accumulation in F1 females (Figure 3), but not in any other generation. ROSI did not elicit any changes in lipid accumulation in males of any generation.

The structure of lipid droplets is maintained by coating proteins such as fat specific protein 27 (Fsp27/CideC), which also participates in metabolism of the droplets (Hall et al. 2009). We analyzed hepatic mRNA levels of Fsp27 and fatty acid transporter protein (FATP) and found that the expression of both is increased in F1 TBT-exposed animals and their F2 and F3 descendants (Figure 4). To further evaluate the hepatic phenotypes, we analyzed the expression of genes implicated in hepatic lipid metabolism. Hepatic PPAR γ and steroid receptor element binding protein 1c (SREBP1c) induce lipogenesis (Pettinelli and Videla 2011), fatty acid synthase (FASN) is a SREBP1c target that promotes fatty acid synthesis (Paulauskis and Sul 1989) and glycerol kinase (GyK) is a PPAR γ target that promotes glycerol uptake and lipogenesis (Tontonoz and Spiegelman 2008). PPAR α and acyl-CoA oxidase (ACOX) are implicated in lipolysis (Abdelmegeed et al. 2011; Reddy and Rao 2006). We observed significant increases in the expression of nearly all lipogenesis- and lipolysis-related genes in males and females from all three generations of derived from TBT animals and in F1 ROSI treated animals, except that males and females differ with regard to changes in PPAR α and GyK in F2 TBT animals (Figure 4). We infer from these data that prenatal TBT exposure of F1 has

transgenerational effects on hepatic lipid metabolism that are reflected by increased hepatic fat storage in subsequent generations (F2 and F3). This is the first demonstration that prenatal obesogen exposure leads to NAFLD and that these changes can be transgenerationally inherited which may have important implications for the increasing incidence of NAFLD.

Discussion

Obesity and related metabolic risk factors such as insulin resistance, hypertension and NAFLD have become a worldwide epidemic (Flegal et al. 2010). These risk factors are strongly associated with the subsequent development of cardiovascular disease and diabetes (de Ferranti and Mozaffarian 2008; Lusic et al. 2008; Reaven 1993). There is an urgent need to understand the mechanisms underlying the predisposition to obesity and related disorders. A growing body of evidence supports the involvement of obesogens that contribute to the burgeoning obesity epidemic (reviewed in Janesick and Blumberg 2011). It should come as no surprise that chemical obesogens exist, because a variety of pharmaceutical drugs (e.g., tricyclic antidepressants, thiazolidinedione antidiabetic drugs, atypical antipsychotics) have been associated with weight gain in humans (reviewed in Janesick and Blumberg 2011). It would be unreasonable to suppose that EDCs targeting the same pathway would not have the same effects. Indeed, we previously showed that the EDC TBT targets the same cellular pathway (PPAR γ) as does the pharmaceutical obesogen ROSI, leading to weight gain, *in vivo* (Grun et al. 2006) and reprogramming of MSC fate to favor the adipogenic compartment at the expense of the osteogenic fate (Kirchner et al. 2010). We recently showed that the fungicide triflumizole also acts through PPAR γ to induce adipogenesis in MSCs and preadipocytes, *in vitro*, through a

PPAR γ -dependent mechanism and promotes increased white adipose depot size and altered MSC programming, *in vivo*, (Li et al. 2012).

Here we showed that prenatal exposure of pregnant F0 animals to TBT caused transgenerational effects that increased WAT depot size, reprogrammed the fate of MSCs predisposing them to become adipocytes and increased hepatic lipid storage and metabolism leading to apparent NAFLD in F1 and subsequent generations. These results persisted until at least the F3 generation. This suggests that the effects of prenatal obesogen exposure can be permanent, thereby leading to widespread changes at the population level over a relatively short time. Our data show the same trend for both, males and females with some relatively modest sex-specific differences. The analyses of the different adipose tissues and the gene expression profiles revealed stronger phenotypes in males than in females in all three generations. In contrast, the NAFLD-like phenotype is somewhat stronger in females than in males. ROSI-exposed mice and their descendants show a more pronounced liver phenotype in females than in males suggesting that there are differences in how TBT and ROSI act between tissues and sexes. It is well known that both TBT and ROSI are PPAR γ activators (Grun et al. 2006; Kanayama et al. 2005), but TBT also activates RXR, which offers potential alternative modes of action. It will be of great interest in future studies to identify the molecular mechanisms underlying these differences.

It is important to note that the doses of TBT used in this study (5.42 nM, 54.2 nM, 542 nM) provide intakes of 0.53, 5.3 and 53 $\mu\text{g}/\text{kg}/\text{day}$ respectively (assuming 10 mL water consumed by a 30g mouse/day). These are, respectively, about 50 fold lower, 5 fold lower and 2 fold higher than the established mouse NOAEL of 25 $\mu\text{g}/\text{kg}/\text{day}$ (Vos et al. 1990). Moreover, they are comparable to the established human tolerable daily intake of 250 ng/kg/day, which was

derived by applying a 100-fold safety factor to the mouse NOAEL (Airaksinen et al. 2010; Fristachi et al. 2009). The degree to which the tolerable daily intake has any relationship to human exposure is uncertain because, to our knowledge, there are no results from moderate or large-scale biomonitoring studies that have established the actual TBT exposure in the population. The few available human biomonitoring studies suggest human serum concentrations of TBT in the range of ~27 nM (Kannan et al. 1999) and for triphenyltin of ~2 nM (Rantakokko et al. 2008). All estimates of the daily human intake of organotins fall far below the tolerable daily intake of 250 ng/kg/day, which raises the important question of how the measured blood levels were achieved. Since the tolerable daily intake is based on food consumption, and organotins have been found in house dust and a variety of other products (Kannan et al. 2010), human exposure may come from multiple sources, only some of which are known. Thus, the dose of TBT we used for these experiments is reasonable and biologically relevant.

It is alarming that the incidence of obesity in U.S. children is high and increasing (Koebnick et al. 2010; McCormick et al. 2010; Taveras et al. 2009) as is the incidence of NAFLD (Mencin and Lavine 2011; Widhalm and Ghods 2010). Diet and exercise continue to be offered as the root cause; however, this cannot explain the results of a recent study, which showed that 8 different species of animals, including pets, laboratory animals and feral rats living in proximity to humans have become obese in parallel with the human obesity epidemic (Klimentidis et al. 2011). The likelihood of this being a chance occurrence has been estimated at about 1 in ten million (Klimentidis et al. 2011). While it is not impossible that each of these 24 different populations of animals has recently increased their food consumption and decreased their exercise levels, it is more reasonable to hypothesize that something else in the environment has changed. Increased exposure to environmental obesogens is one possibility. Our

demonstration that prenatal exposure to TBT leads to increased adipogenesis, reprogramming of MSCs to favor the adipocyte lineage and development of fatty livers together with the observation that these effects are passed on to the F2 and F3 generations, suggests that the effects of obesogen exposure may be even more damaging than had previously been appreciated. It will be of great interest to identify the mechanisms through which TBT exerts transgenerational effects on adipogenesis and NAFLD and the extent to which alterations in stem cell fate and function are involved.

Skinner and colleagues first showed that high doses of the fungicide vinclozolin can lead to transgenerational effects on male fertility, tumors, prostate disease, kidney diseases and immune abnormalities (Anway et al. 2005) resulting from epigenetic changes in gene expression (Skinner 2011a). Since this landmark study, many other studies have demonstrated multigenerational effects of EDCs on a variety of organ systems (reviewed in Skinner 2011b). While early studies demonstrating transgenerational inheritance of EDC effects elicited by high doses of single chemicals, more recent studies showed that high doses of a variety of chemicals (fungicides, pesticide mixture, plastic mixture, dioxin, and a hydrocarbon mixture) could also elicit transgenerational effects (Manikkam et al. 2012; Nilsson et al. 2012). It was recently shown that administration of bisphenol A to achieve plasma levels similar to those measured in humans could cause transgenerational alterations in genes and behavior (Wolstenholme et al. 2012b). Ours is the first study to demonstrate transgenerational effects of developmental exposure to environmentally-relevant concentrations of an obesogen on adipogenesis, MSC programming and hepatic fat accumulation. These results have important implications both for understanding the obesity epidemic and for the ongoing discussion about the dangers of EDCs. If, as we expect, our model applies to humans, then prenatal obesogen exposure could

permanently reprogram the metabolism of exposed individuals, predisposing them toward weight gain, particularly in conditions of caloric excess. The transgenerational inheritance of obesogen exposure, particularly the increased magnitude of the effects in the F3 generation raises the stakes in the ongoing debate regarding what weight of evidence is required for regulatory agencies to take action to reduce EDC exposure.

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Figure Legends

Figure 1. Transgenerational effect of TBT on adipose depots from F1, F2 and F3 male mice. A) Adipose tissue weights are represented as the percentage of total body weight. B) Relative adipocyte size in epididymal, peri-renal and subscapular white adipose tissues, and lipid accumulation for subscapular brown adipose tissue. C) Number of adipocytes per fat depot assayed by total DNA quantitation. All data are expressed as the average from 9-18 animals \pm SEM. We used one-way ANOVA with Dunnett's posthoc test for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Figure 2. Gene expression profile of MSCs from F1, F2 and F3 male mice. The relative mRNA levels of specific transcripts for adipogenic (A) or osteogenic (B) differentiation were assayed by QPCR in undifferentiated MSCs, with the expression of each target gene normalized to β -Actin. All data are expressed as average fold change \pm SEM in 3 biological replicates assayed in duplicate. We used unpaired t-tests for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Figure 3. Transgenerational effects of TBT on hepatic lipid accumulation. Representative histological sections of frozen livers stained with Oil Red O and hematoxylin in males from F1, F2 and F3 A) male and B) female mice. At least five animals per group were analyzed and representative pictures are shown. The bar = 50 μ m.

Figure 4. Transgenerational effects of TBT on markers of hepatic lipid metabolism in F1, F2 and F3 mice. Relative mRNA levels of PPAR γ 2, SREBP1c, GyK and FASN (lipogenic markers); PPAR α , ACOX (lipolytic markers) and Fsp27, FATP (lipid droplet markers) were evaluated in total RNA from A) male and B) female mouse livers. All data are expressed as average fold

change \pm SEM in 3 biological replicates assayed in duplicate. We used unpaired t-tests for statistical analyses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Chamorro-Garcia et al. Figure 1

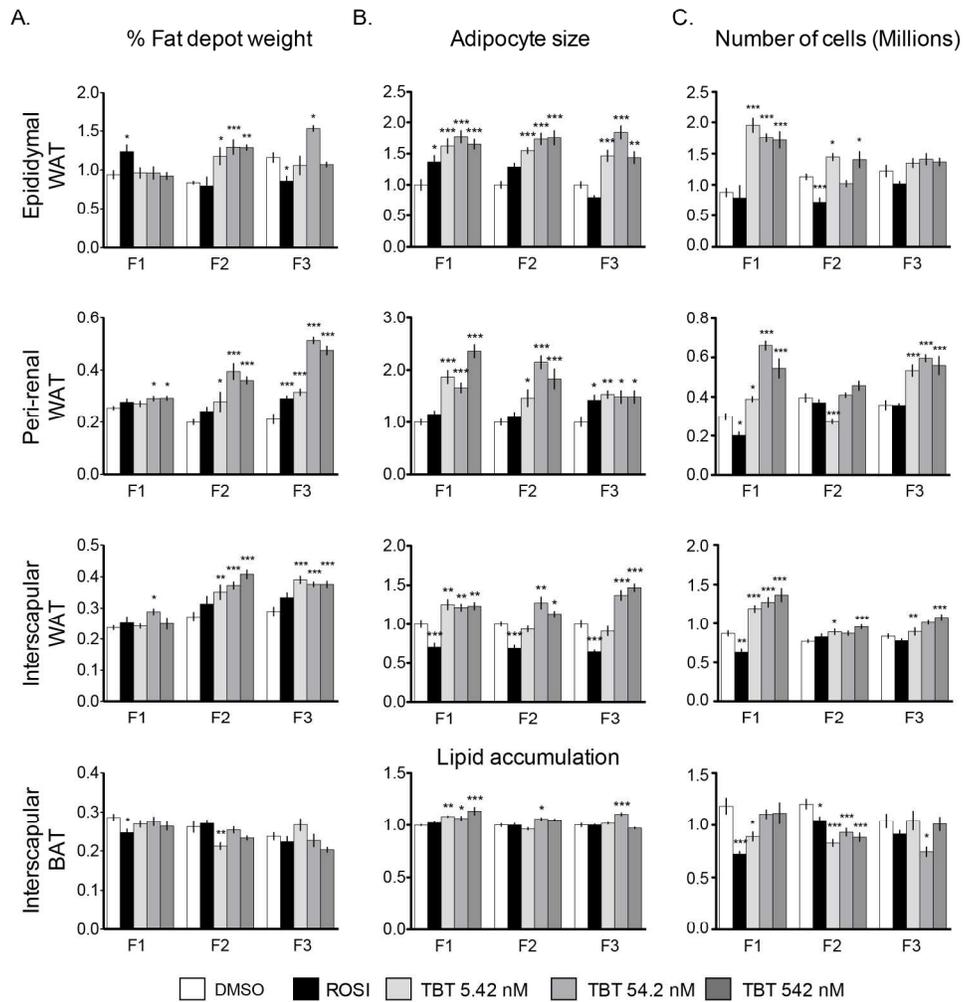


Figure 1
190x212mm (300 x 300 DPI)

Chamorro-Garcia et al. Figure 2

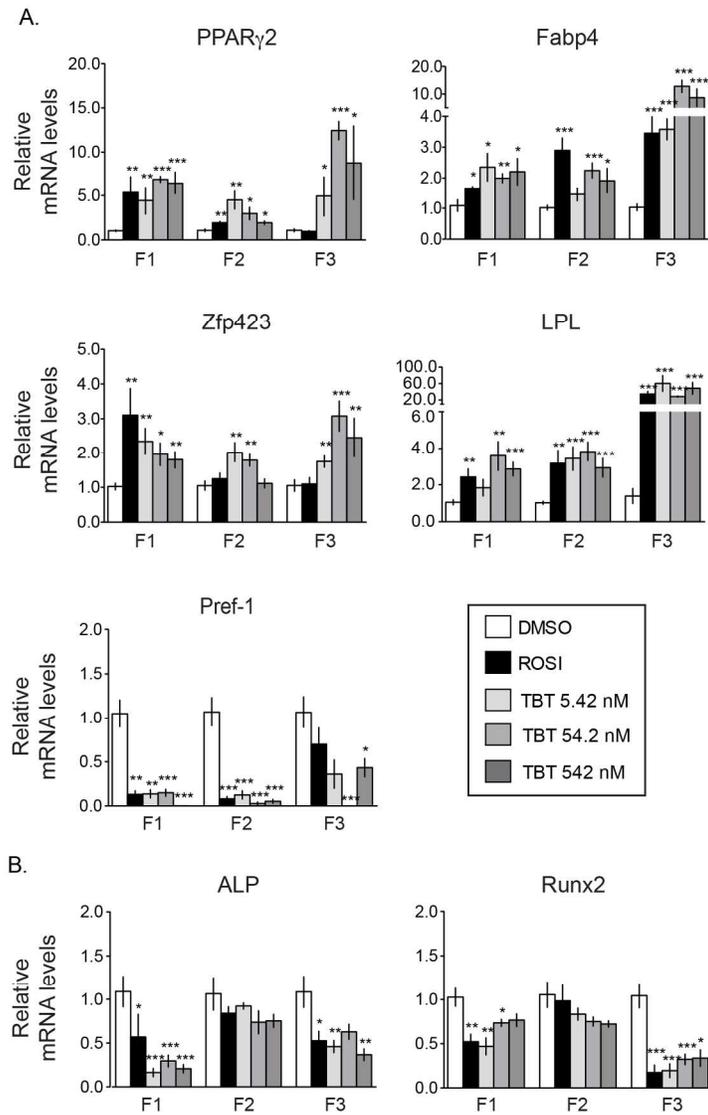


Figure 2
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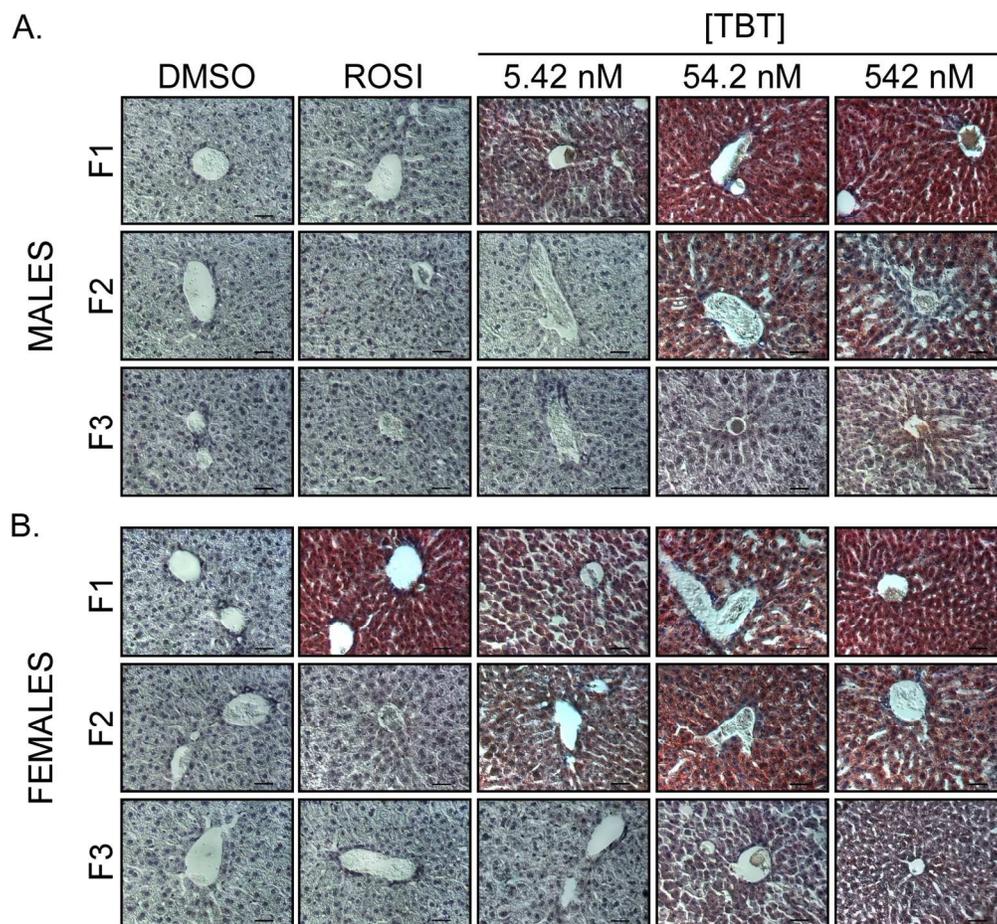
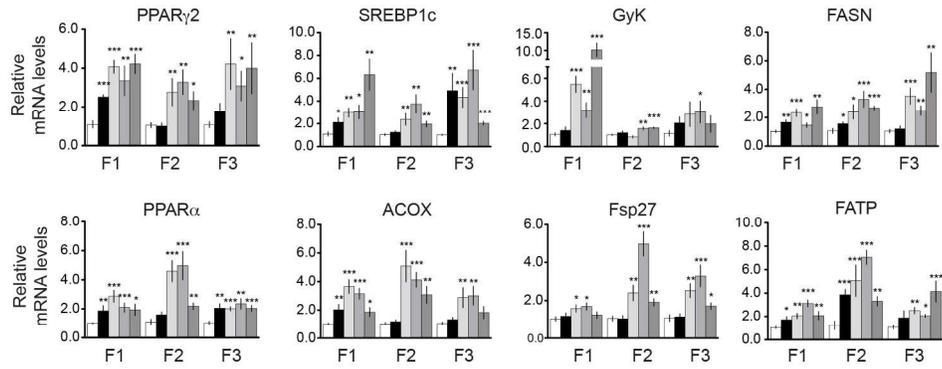


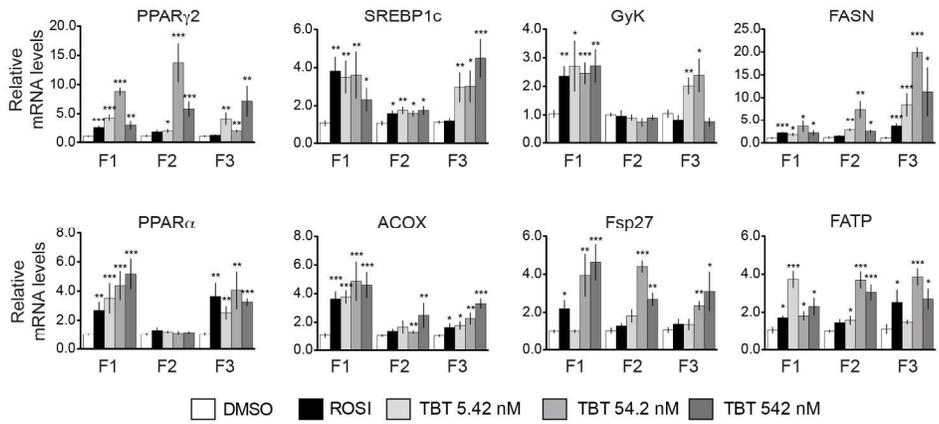
Figure 3
148x136mm (300 x 300 DPI)

Chamorro-Garcia et al. Figure 4

A. Males



B. Females



□ DMSO ■ ROSI □ TBT 5.42 nM □ TBT 54.2 nM ■ TBT 542 nM

Figure 4
200x193mm (300 x 300 DPI)