Specific Aims

Evidence from labs around the world supports the idea that environmental factors, when encountered during critical time windows, are manifested in health consequences later in life. Early exposure to endocrine disrupting chemicals (EDCs) is an emerging factor that perturbs an individual's metabolic setpoint and increases his/her propensity towards obesity. Our published work identified tributyltin as an environmental "obesogen" that programs mice to prematurely accumulate adipocytes during development, via activation of the peroxisome proliferator activated receptor gamma (PPARγ) to alter the fate of multipotent mesenchymal stem cells (MSCs). Other obesogens have been identified but evidence for their mechanisms of action is scant. Preliminary results demonstrate that bisphenol A diglycidyl ether (BADGE) induces adipogenesis in both mouse and human MSCs, suggesting that it may act as an obesogen. Intriguingly, although BADGE has been described as a PPARγ antagonist at high (100 μM) concentrations, we found that BADGE induces adipogenesis at nanomolar levels in MSCs through a pathway that appears not to involve PPARγ. *This R21 application will test the hypothesis that BADGE acts as an obesogen, in vivo, through a molecular pathway that is parallel to or downstream of PPARγ* and will establish effective exposure strategies and explore candidate pathways of action. The following specific aims are proposed to test our hypothesis:

Aim 1: Does BADGE exposure predispose mice to increased adipogenesis and obesity and if so, does it alter lineage allocation in the MSC compartment? - It is currently controversial whether BADGE promotes or inhibits adipogenesis. Our results suggest that at low, environmentally-relevant doses (nM), BADGE promotes MSC differentiation into adipocytes. To ascertain whether BADGE can act as an obesogen, in vivo, we will test the effects of prenatal, perinatal, and adult exposure to BADGE on adipogenesis, body mass, fat depot size, blood biochemistry and the allocation of MSCs to the adipocyte lineage compared with other potential fates for these cells.

Aim 2: What molecular pathway does BADGE act through to influence adipogenesis and obesity? - We will employ specific chemical antagonists and lentivirus-delivered shRNA-mediated knockdown of candidate genes in pathways known to be important in adipogenesis for their ability to block the adipogenic activity of BADGE in MSCs.

The proposed work will allow us to make rapid progress in establishing whether the high volume chemical, BADGE, is obesogenic in vivo. By investigating the role of BADGE in adipogenesis, we will be able to more clearly understand the developmental pathways controlling the prenatal programming of MSC fate and how obesogen exposure alters this fate. Furthermore, by investigating a chemical that is adipogenic but PPARγ-independent, we will be able to elucidate pathways downstream of PPARγ which are biologically relevant.

Significance and Potential Impact – The contribution of EDCs to the obesity epidemic has recently been recognized. Early prenatal exposure to obesogenic EDCs can program the fetus towards an obese phenotype where adipose tissue is resistant to reduction in cell number or size. Therefore, the knowledge gained about obesogens will inform preventative measures to reduce exposure at critical periods during life. BADGE is a chemical that appears to be ubiquitous in humans and occurs at the levels (500 pM-50 nM) that we find promotes adipogenesis in MSCs. Therefore, the potential impact of identifying BADGE as an obesogen, in vivo, is high. We have proposed doses and routes of exposures that are relevant to human exposures and are in accordance with the types of studies needed by regulatory agencies for risk assessment purposes (multiple, oral doses at different life stages). The successful completion of this research will make important contributions to understanding the maternal programming of obesity, how obesogens affect this process, and what is the contribution of altered stem cell fate.

Innovation – The obesogen hypothesis has opened a new area of research into obesity by connecting endocrine disruptor research with developmental origins of disease. We have made the novel observation that BADGE induces adipogenesis in MSCs through a pathway not dependent on activity of PPARγ.

Approach – We will use animal models to test the hypothesis that BADGE acts as an obesogen, in vivo, and a multipotent stem cell model to investigate the pathways thorough which BADGE might act to promote adipogenesis and obesity.

Background and Significance

Obesity and obesity-related disorders such as diabetes and cardiovascular disease are a worldwide public health epidemic, particularly in the US. The major factors driving obesity are most often ascribed to genetics (1) and behavioral factors such as smoking (3), excessive consumption of alcohol (4) and food (5), stress (6) and sedentary lifestyle (7). However, environmental factors, such as exposure to xenobiotic chemicals, are under-studied compared with diet and lifestyle, in the development of obesity. Since the magnitude of the burden on the U.S. healthcare system exceeds $147 billion annually, a detailed study of the role of environmental chemicals on the etiology of obesity, particularly via maternal exposure, is timely and important.

In 2006, we proposed the existence of endocrine disrupting chemicals (EDCs) that could influence adipogenesis and obesity which could be important, yet unsuspected players in the obesity epidemic. "Obesogens" are defined functionally as chemicals that promote obesity by increasing the number of fat cells or the storage of fat into existing fat cells. Obesogens can also act on adipocytes indirectly by changing the basal metabolic rate, by shifting energy balance to favor the storage of calories, and by altering hormonal control of appetite and satiety (reviewed in 8, 9-12). While the obesogen hypothesis was initially controversial, many obesogenic chemicals have been identified in recent years, underscoring the relevance of this novel model. Estrogens such as diethylstilbesterol and genistein (13), organotins such as tributyltin (14), perfluorooctanoates (15), and bisphenol A (16) are known to be obesogenic in animals. Phthalates correlate with increased waist diameter in humans (17).

A variety of chemicals have been shown to increase adipogenesis in pre-adipocyte cell lines such as murine 3T3-L1 cells (14, 18-23), and in primary multipotent mesenchymal stem cells, now called multipotent stromal cells (MSCs) (2, 24, 25). To date, the only obesogen with a known pathway of action is tributyltin (TBT), which activates the peroxisome proliferator activated receptor gamma (PPARγ) to promote adipogenesis by biasing the MSC compartment toward the adipocyte lineage (2). Hence, there is a very large knowledge gap concerning the number of potential obesogens and their modes of action.

Bisphenol A diglycidyl ether (BADGE), produced by reacting bisphenol A (BPA) and epichlorhydrin, is used as an intermediate in the manufacture of epoxy resins, paints and also as a coating on food cans and food storage vessels (26). BPA and BADGE are present in other commonly used products including beverage containers, baby bottles and dental composites (26). They migrate from container linings into foods, and are routinely ingested (27, 28), raising questions concerning their potential adverse affects on human health. There are many reports on the estrogenic and anti-androgenic effects of BADGE using in vitro assays (29-31); however the manufacturers of BADGE dispute any reproductive or endocrine disrupting effects (26).

The obesogenic properties of BADGE are yet to be thoroughly investigated. BADGE was identified as a pure antagonist of PPARγ with an affinity of ~100 μM (32) and shown to inhibit adipocyte differentiation induced by PPARγ ligands in murine 3T3-L1 and 3T3-F442A preadipocytes (33). In contrast, others (32) reported that BADGE activated PPARγ and induced its nuclear localization in a human umbilical vein endothelial ECV304 cell line. BADGE activated PPARγ to suppress tumor necrosis factor alpha production in a macrophage cell line (34). The overall conclusion is that the effects of BADGE on PPARγ, adipogenesis and other cellular processes may be tissue or cell-type specific. As noted in Preliminary Studies, we found that BADGE induces adipogenesis and the expression of adipocyte genes in MSCs in a PPARγ-independent manner. This leads us to hypothesize that BADGE in an obesogen. Considering the likely widespread exposure to BADGE in the population, it is important to know whether BADGE acts as an obesogen and through what pathways.

3a - **Significance of the proposed research** - 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, the study of environmental factors in obesity is only beginning and the mechanisms remain largely unknown. Our published work showed that TBT is involved in critical steps of adipogenesis in vitro and in vivo though its actions on PPARγ and that TBT exposure predisposes MSCs to become adipocytes by epigenetic imprinting into the memory of the MSC compartment (2). The proposed work will determine the fate of MSCs from various locations in the body and determine if circulating MSCs (e.g., from the bone marrow) actually colonize fat depots during development and early adulthood. By unraveling the complex modulation of developmental pathways controlling the prenatal programming of MSC fate, our

research will make important contributions to understanding the development of obesity and how obesogens affect this process. The proposed research builds on our preliminary studies to establish the mode of action and overall effects of BADGE exposure on adipogenesis and fat deposition in a mouse model. This research will make an important contribution to understanding the role of xenobiotic compounds on obesity and on which compounds should be the focus of regulatory efforts to ameliorate the obesity epidemic. The successful completion of the proposed research will enable us to refine the pathways of action for study in a future R01 application.

3b- Innovation – Our obesogen hypothesis has opened a new area of research into obesity by connecting endocrine disruptor research with developmental origins of disease. We made the novel observation that BADGE induces adipogenesis in MSCs through a PPARγ-independent pathway. This challenges the paradigm that BADGE is a PPARγ antagonist (33). In addition, this addresses the idea that adipogenic potential does not necessarily imply activation of PPARγ, or increase in PPARγ expression.

The use of MSCs as models for EDC research is innovative. MSCs are plastic-adherent fibroblasts found in several tissues, which have the ability to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and neurons, in vitro (35). MSCs have emerged as a model to study adipogenesis because they exhibit gene expression profiles during differentiation comparable to that of other in vitro models, such as 3T3-L1 cells (36). However, a key advantage of MSCs is that they enable the study of the earliest events in committing cells to the adipogenic pathway.

3c - Approach – We will use animal models to test the hypothesis that BADGE acts as an obesogen, in vivo, and a multipotent stem cell model to investigate the pathways thorough which BADGE might act to promote adipogenesis and obesity.

Suitability of our laboratory for the proposed research - Our laboratory studies the role of nuclear hormone receptors and their ligands in embryonic development and the regulation of physiology. We have more than 19 years of experience in this field and have published significant papers in the areas of ligand identification (37-39), orphan receptor characterization (38-40), receptor modulation by xenobiotic compounds (39, 41-44), microarray analysis of receptor target genes (45) and the role of nuclear receptors in developmental processes (2, 44, 46-48). We identified TBT as an RXR-PPARγ ligand, and showed that prenatal exposure leads to enhanced lipid accumulation and increased fat depot size (14). We successfully established MSCs as a model to test the effects of obesogen exposure in vitro and in vivo and showed that TBT altered the fate of MSCs to favor the adipocyte lineage (2). The proposed research will draw upon many of the tools we used to study TBT to determine whether BADGE induces adipogenesis in mouse and human MSCs in vivo.

Preliminary studies

Converging lines of evidence from epidemiological studies and animal models show that the origins of obesity lie not only in the interaction between genes and traditional adult risk factors such as diet and exercise, but also in the interplay between genes and the fetal and early postnatal environment. We propose that exposure to environmental obesogens is superimposed on these conditions to promote the development of obesity (8-10). BADGE is a chemical very widely used in commerce and, although, precise exposure data are scant, it has been suggested that human exposure, probably via dietary sources, is likely to be widespread (26, 49). Our preliminary results are summarized here and key findings presented below. Briefly, we found that:

- BADGE induces adipogenesis in human and mouse MSCs (**Fig 1, 2**)
- BADGE does not activate or antagonize PPARγ at up to 10 μM; therefore, BADGE is unlikely to act as a ligand for the RXR-PPARγ heterodimer at doses that could be encountered, *in vivo* (**Fig 3**).
- BADGE-induced adipogenesis is not affected by treatment with the PPARγ antagonist GW9662, suggesting that BADGE acts independently, or downstream of PPARγ (**Fig 4**).
- BADGE does not affect the ability of the osteogenic cocktail to induce osteogenesis, confirming that it acts in parallel to or downstream of PPARγ (**not shown**).

BADGE is thus a strong candidate for an obesogen that may contribute to the obesity epidemic. We believe it important to establish whether BADGE acts as an obesogen, *in vivo* and to refine our understanding of the molecular pathways through which BADGE acts to influence adipogenesis.

BADGE exposure induces human and mouse MSCs to become adipocytes - Treatment of hMSCs or mMSCs with standard adipogenic induction medium MDII (IBMX, dexamethasone, indomethacin and insulin) (2) caused about 15% (mMSCs) or 30% (hMSCs) of the cellular area to be covered by lipid droplets (Fig 1). Surprisingly, considering its pro-adipogenic properties, in vivo (50), BPA was unable to induce adipogenesis at up to 100 µM (Fig 1A); higher concentrations were cytotoxic. BADGE significantly induced adipogenesis at 50 pM in human and 100 pM in mouse MSCs (Fig. 1B). The activation is dose-dependent and reaches an apparent plateau at 100 µM BADGE.

100 nM BADGE gave similar effects on adipogenesis to 500 nM of the potent PPARγ activator rosiglitazone (ROSI) (Fig 2 left). QPCR measurement of mRNA levels at the end of the 14-day induction/differentiation period revealed a gene expression profile consistent with enhanced adipogenesis in the MSCs exposed to BADGE (Fig. 2 right). The early adipogenesis marker aP2 (>3 fold), the late markers LEP (~6 fold) and LPL (>8 fold) were significantly increased, whereas the adipogenesis inhibitor Pref-1 was decreased (~2.5 fold) when MDII was supplemented with 500 nM ROSI or 100 nM BADGE (Fig. 2). ROSI significantly induced PPARγ mRNA, but induction by BADGE did not reach statistical significance (Fig 2). Neither ROSI nor BADGE induced the late marker, ADIPOQ in mouse MSCs (Fig 2) and only BADGE induced ADIPOQ expression in human MSCs (not shown).

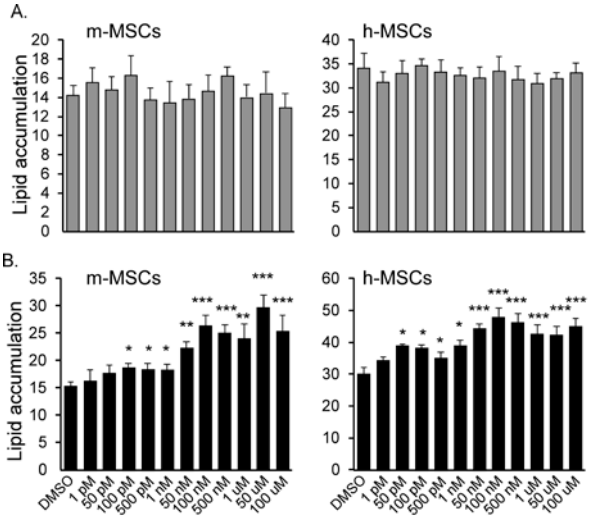

Figure 1 – BADGE (B), but not BPA (A) induces adipogenesis in mouse and human MSCs. *Human or mouse MSCs were treated with the indicated doses of (A) BPA or (B) BADGE, or DMSO vehicle in standard adipogenesis assays (2). * $p \leq 0.05$, **, $p \leq 0.01$, *** $p \leq 0.001$.*

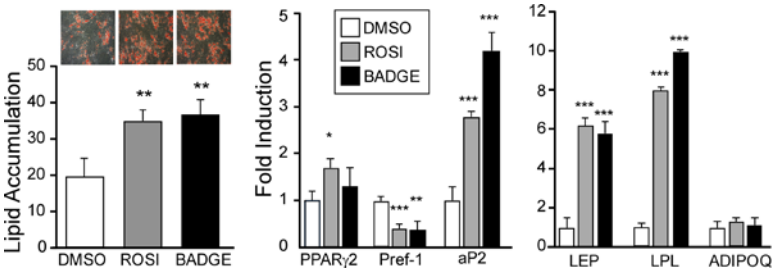

Figure 2 – BADGE and ROSI induce adipogenesis and expression of adipocyte marker genes in mouse MSCs. *MSCs were treated with 500 nM ROSI, 100 nM BADGE or 0.1% DMSO in standard adipogenesis assays (2). The left panel shows the amount of adipogenesis induced by the treatment. The right panels show QPCR analysis of marker genes. * $p \leq 0.05$, **, $p \leq 0.01$, *** $p \leq 0.001$.*

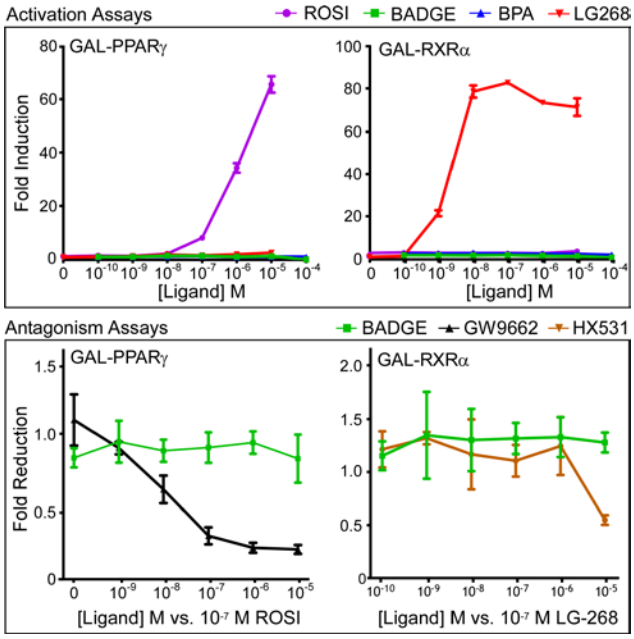

Figure 3 – BADGE has no effect on RXR or PPARγ in transient transfection assays. *The ability of BADGE to activate or antagonize activation of PPARγ or RXRα was tested using transient transfections. BADGE neither activated nor antagonized either receptor, nor did BPA. ROSI - PPARγ activator, LG268 - RXR activator, GW9662 -* PPARγ *antagonist, HX531 – RXR antagonist.*

BADGE-induced adipogenesis is PPARγ-independent - Many environmental EDCs mimic natural lipophilic hormones that act through members of the nuclear hormone receptor superfamily (51). Because PPARγ is believed to be the master regulator of adipogenesis (52), and BADGE has been shown to activate PPARγ in some cell types (32, 34, 53), we hypothesized that BADGE exerted its effect on the adipogenic capacity of MSCs by activating PPARγ. However, whereas ROSI could fully activate human PPARγ in transient transfection assays, neither BADGE, nor BPA had any effect on activation of PPARγ, or its heterodimeric partner, RXR (Fig. 3). BADGE has been described as a PPARγ antagonist and adipogenesis inhibitor at very high concentrations (100 µM) in 3T3-L1 preadipocytes (33). We tested the ability of BADGE to antagonize PPARγ and RXRα and found that it was unable to antagonize either receptor at concentrations below 10 µM (higher concentrations were cytotoxic) (Fig 3).Therefore, we conclude that neither BADGE nor BPA activate or antagonize the RXR-PPARγ heterodimer in transient transfection assays.

We next tested whether inhibiting PPARγ with the potent antagonist GW9662 had any effect on BADGE induction of adipogenesis in MSCs. GW9662 almost completely inhibited the ability of ROSI to promote adipogenesis, but had no effect on the ability of BADGE to promote adipogenesis, even at 5 fold molar excess (Fig 4). Hence, BADGE specifically facilitates adipose conversion of MSCs but this effect cannot be blocked by antagonizing PPARγ (in contrast to TBT or ROSI).

The reciprocal relationship between adipocyte and osteocyte commitment and differentiation is well documented. It involves a shift in the flow of mesenchymal precursor cells from osteoblastic to adipogenic lineages, which is mediated by PPARγ (54). We found that ROSI strongly inhibited bone differentiation in MSCs as measured by matrix calcification and the expression of mRNA encoding bone markers such as alkaline phosphatase (ALP) and osteocalcin (OST) (2). BADGE did not alter matrix calcification or the expression of any bone markers (data not shown). This suggests that in contrast to what we found with the EDC TBT (2), BADGE does not promote or facilitate adipogenic differentiation to the detriment of osteogenic differentiation in MSCs.

Considering the ability of BADGE to promote adipogenesis in MSCs, its failure to activate or antagonize PPARγ or alter the ability of MSCs to undergo osteogenesis and the inability of GW9662 treatment to block BADGE-induced adipogenesis, we conclude that BADGE induces adipogenesis independently of PPARγ. The pathway could be parallel to, or downstream of PPARγ and will be investigated in Aim 2.

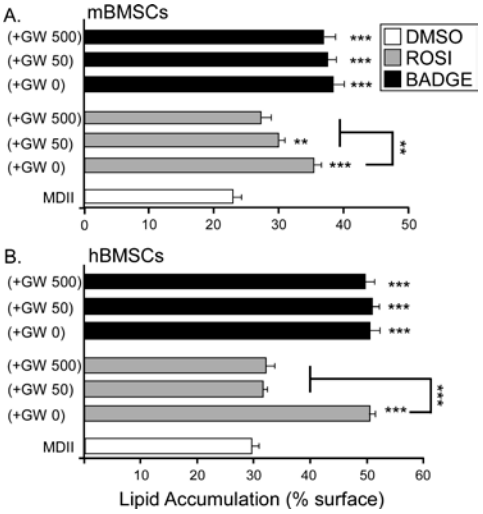

Figure 4 – BADGE induces adipogenesis in a PPARγ-independent manner. *Adipogenesis was induced in mouse(A) or human (B) MSCs using standard conditions (2) plus 500 nM ROSI or 100 nM BADGE in the presence of DMSO, 50 or 500 nM GW9662 After staining with Oil Red O, and quantitation with Image J (2), the data were expressed as average lipid accumulation in N=9 replicates ± SEM relative to vehicle (DMSO) controls. Asterisks illustrate significant differences from MDII * p ≤<0.05; **, p≤0.01; ***, p≤0.001).*

D. Research Design and Methods

Introduction: Our preliminary studies show that BADGE induces the differentiation of mouse and human MSCs into adipocytes in vitro and promotes the expression of adipocyte marker genes. This process is not blocked by the PPARγ antagonist GW9662, suggesting that BADGE acts on a pathway that is downstream of PPARγ, or parallel to it. *Our specific hypothesis is that BADGE acts as an obesogen, in vivo, through a pathway parallel to, or downstream of PPARγ.* Below we propose experiments to test this hypothesis and identify the molecular pathway through which BADGE acts.

Aim 1: Does BADGE exposure predispose mice to increased adipogenesis and obesity and if so, does it alter lineage allocation in the MSC compartment?

Rationale and hypothesis: We have established that BADGE causes human and mouse MSCs to differentiate into adipocytes, induces the expression of adipocyte marker genes (**Figs 1,2**) and that this process is not blocked by the PPARγ antagonist GW9662 (**Fig 4**). A key question is whether the levels at which we find effects in MSCs are environmentally relevant. Although there are currently no routine biomonitoring studies underway for BADGE anywhere in the world, BADGE intake in Europe was "guesstimated" by computer modeling at 0.16 µg/kg/day (~470 pM) (26). The Environmental Working Group informally surveyed 22 people the US for exposure to 70 industrial chemicals and found an average serum BADGE level of 17.3 ng/mL (~51 nM) (49); and Health Canada estimated the maximum daily intake of BADGE from infant soy formula at 22 ug/kg/day (~65 nM) (28). Therefore, whether one accepts the low or high estimates, the concentrations we have used in MSCs are within the range of human exposure. *We hypothesize that BADGE will act as an obesogen in vivo at environmentally relevant (~500 pM-100 nM) concentrations.*

Experimental design, expected results and analysis: Several in vivo studies addressing the effects of BADGE on body weight have been reported (55-58) that utilized exposure levels of hundreds of mg/kg, an unrealistically high dose compared with predicted human exposures. Although animals lost weight in those studies there is ample precedent for chemicals causing weight gain at low doses, but weight loss at high doses (13). Hence, we might reasonably anticipate BADGE to cause weight gain at low doses instead of weight loss.

Pilot study: We will employ chronic exposure via the drinking water in two standard mouse models (3 animals/dose), C57BL/6J (to facilitate future use of mutant mouse models) and CD-1 (an outbred strain that

may be more relevant to human outcomes). Doses chosen will deliver daily doses of 500 pM (the low human estimate), 50 nM (representing an average human exposure), 100 nM (to parallel in vitro studies), 500 nM (intermediate dose), and 5 μM (high dose). Positive controls will be animals exposed to 500 nM of rosiglitazone (ROSI) based on results obtained in previous work performed in our laboratory (2) and which also parallels the dose used for *in vitro* exposure to the MSCs. Compounds will be diluted in 0.5% carboxymethyl-cellulose (CMC) vehicle to minimize adhesion to the bottle walls. Pilot studies will be performed using chronic 4-week exposure in 8 week old animals to test the effects of adult exposure on adipose depot size, gene expression in adipocytes and the ability of MSCs to be differentiated into adipocytes as described in more detail below.

Developmental exposures: We expect to obtain LOEL from the pilot experiments and will use 0.1x, 1.0x and 10x LOEL in subsequent studies. Three dosing regimens (via drinking water) will be used 1) prenatal exposure from plug detection through birth, 2)postnatal exposure of the dams from birth until weaning, and 3) young adult exposure (from weaning to 8 weeks). For prenatal exposure assays, the dams will be exposed to the chemicals beginning 1 week before breeding to ensure that the offspring will be exposed to the chemicals from the embryonic day 0 until birth. Prenatally-exposed newborns will be fostered to unexposed dams to distinguish the influence of prenatal exposure, vs. exposure during lactation. Comparison of the results obtained from these 3 dosing regimens will allow us to identify sensitive periods to be refined in future studies.

After weaning, control or exposed pups will be maintained on normal low-fat rodent chow with *ad libitum* food access. Food intake will be recorded every three days by weighing the food. Mice will be euthanized at 12 weeks with terminal blood samples taken for glucose, lipid and adipokine measurements. Fat depots will be dissected, weighed and divided in three portions: one will be fixed and prepared for histology, another one will be used to study gene expression of adipose tissue markers (aP2, PPARγ2, PEPCK, LPL, leptin and ADIPOQ), and the last portion will be used for MSC preparation. MSCs will be harvested from bone marrow and adipose depots as previously described (2). To investigate the effect of treatments on lineage allocation, MSCs from control or treated animals will be exposed *in vitro* to adipocyte or bone induction cocktail (2) plus 500nM ROSI or 100nM BADGE. The adipogeneic and osteogenic differentiation assays will identify changes in lipid or calcium accumulation (revealed by staining cells with Oil Red O or Alizarin Red S, respectively) and corresponding changes in key markers of adipogenic (aP2, PPARγ2, PEPCK, LPL, leptin and ADIPOQ,) or osteogenic (alkaline phosphatase and osteopontin) pathways. The results will be compared to the *in vitro* DMSO control baselines. In parallel experiments, animals will be followed for up to 6 months with regular monitoring of weight, blood biochemistry and longitudinal measurement of fat vs. lean mass (in the UCI Center for Functional Oncoimaging). Data will be analyzed using a Kruskal-Wallis non-parametric test, followed by a Dunn's post-hoc test to determine the significance of the difference in relative mRNA abundance among groups with different treatments (2). $P< 0.05$ will be considered statistically significant.

We expect to observe increased adipogenesis and fat depot size in some of the treatment regimens. Using 3 different exposure windows will be informative for inferring effects of human exposure, particularly considering the levels of BADGE expected to be ingested together with soy formula (28). If we find effects from prenatal or early life exposure, subsequent experiments aimed at identifying epigenetic alterations underlying changes in MSC fate will be considered along the lines of those underway in our lab for TBT (2).

Potential pitfalls and alternative approaches: One obvious pitfall is that BADGE might have no effect, in vivo. We consider this result unlikely since there are no examples of chemicals known to induce adipogenesis in MSCs that do not cause increased adipogenesis and weight gain, in vivo, at appropriate doses. Irrespective of the outcome of these experiments, it is critically important to determine whether chemicals that induce adipogenesis in vitro, act similarly in vivo. We have proposed dosing and routes of exposures that are relevant to human exposures and are in accordance with the types of studies needed by regulatory agencies for risk assessment purposes (multiple, oral doses at different life stages). Future studies aimed at uncovering effects on detailed metabolic endpoints, glucose metabolism, diabetes, and development of cardiovascular disease will be the subject of an R01 application based on the successful outcome of these studies.

Aim 2: What molecular pathway does BADGE act through to influence adipogenesis and obesity? –

Rationale and hypothesis: Two key pieces of preliminary data establish the need for investigating the pathway(s) of BADGE action: 1) BADGE neither activates nor antagonizes PPARγ or RXR in cell culture (**Fig 3**) and 2) BADGE induction of adipogenesis in human and mouse MSCs is not blocked by the PPARγ antagonist GW9662 **(Fig 4**). Since PPARγ is indispensable for adipogenesis (59, 60) *we hypothesize that*

BADGE acts through a pathway that is parallel to, or downstream of PPARγ. Therefore, we will begin with a candidate gene approach to identify pathways targeted by BADGE and follow this with more broad-based screening to more precisely characterize the pathways involved.

Experimental design, expected results and analysis: We will employ specific chemical antagonists and shRNA-mediated gene knockdown to test candidate pathways known to be important in adipogenesis for their ability to block the adipogenic activity of BADGE in MSCs. BADGE appears not to act through PPARγ; however, PPARγ is indispensable for adipogenesis (61). Since GW9662 and shRNA cannot generate complete loss-of-function, we will generate PPARγ-deficient MSCs by crossing the floxed PPARγ mouse (DHHS #E151-2003/0) with an adipocyte-specific Cre mouse (DHHS #E-151-2003/2) available from the NIH mouse model repository, following our standard methods (2). PPARγ$^{-/-}$ MSCs will be treated with ROSI, BADGE or vehicle to test the dependence of BADGE on PPARγ. Published findings suggest that these cells will be unable to produce adipocytes (61). A finding that BADGE induces adipogenesis in the absence of PPARγ would establish a new paradigm for adipocyte differentiation. If BADGE is unable to induce adipogenesis PPARγ$^{-/-}$ MSCs, then we will infer that BADGE acts parallel to or downstream of PPARγ, but requires the latter for full effect.

BADGE has been proposed to act as an estrogen and anti-androgen, hence we will test its effect on the estrogen androgen and glucocorticoid pathways using specific chemical agonists and antagonists as in **Fig 4** for their ability to augment or inhibit BADGE-mediated adipogenesis. Open Biosystems provides readymade lentivirus based shRNAs for nearly all human and mouse genes. Lentivirus delivery is known to be effective in MSCs (62, 63) and we will test the effects of knocking down expression of candidate genes on BADGE-induced adipogenesis. Briefly, MSCs are plated in 12-well dishes, grown to confluency, infected with test, or control lentivirus particles in duplicate and the ability of 100nM BADGE, 500 nM ROSI or vehicle to induce adipogenesis tested in standard assays (2). Constructs showing inhibition of BADGE-mediated adipogenesis will be selected for further study. We will test their effects on BADGE-induced expression of adipocyte markers by QPCR as for aim 1. Since GW9662 does not inhibit BADGE-induced adipogenesis (**Fig 4**), the ideal candidate gene(s) would inhibit BADGE- but not ROSI-mediated adipogenesis. We are well acquainted with the literature concerning adipocyte differentiation and recent microarray and ChIP studies that identified new genes in adipogenic differentiation (52, 61, 64-68). Rather than elaborate a long laundry list of candidate genes, we will start with major pro-adipogenic genes such as C/EBPα,ß,,δ; SREBP1c, Krox20, KLF4,5,KLF15, Zfp423, CBP/p300, PGC-1α, LXR and then major negative regulators such as GATA2,3, Wnt, MEK/ERK, KLF2, SIRT1, SMART/NCoR, RIP140 (52), and test other candidate genes known to be downstream of PPARγ (64, 65, 67, 68) depending on the initial findings. The results of this analysis should give us a clear idea of which pathways are involved and what future directions should be taken.

Potential pitfalls and alternative approaches: One criticism of this aim is that it is partly dependent on the results of aim 1. If we do not find that BADGE is an obesogen, in vivo, then the impact of experiments proposed in this aim would be diminished. However, identifying PPARγ-independent adipogenic pathways has the potential to improve our understanding of adipogenesis, *per se*. While open-ended experiments such as those proposed above can be criticized as “fishing expeditions”, we believe that they are essential to inform and refine future efforts to fully understand BADGE action and adipogenesis in general. Moreover, such high risk, high reward approaches are ideal for the R21 mechanism.

If we are unsuccessful identifying a candidate pathway mediating BADGE action, or if a deeper analysis of identified pathways is desired, we will undertake a more broad-based approach. Transcriptome profiling using microarray or RNA-seq analysis of genes regulated by BADGE compared with vehicle will be ideal for this purpose. Mouse MSCs would be preferred to moderate the number of biological replicates required to demonstrate robust differences in gene expression suitable for pathway analysis. Briefly, mouse MSCs (from 5 different mice) are cultured under our standard conditions (2), treated with 100 nM BADGE or vehicle for an additional 3 days, then harvested for mRNA preparation. QPCR analysis will verify that adipogenesis is proceeding as measured by expression of the early markers aP2 and Pref-1 (which should be up- and down-regulated respectively). If significant changes in expression of these genes is found, the RNA will be submitted to the UCI microarray core for probe preparation, microarray analysis or RNA-seq. Genes significantly induced by BADGE will be validated by QPCR and then tested via the shRNA lentivirus approach. We have experience with microarray analysis (45) and do not anticipate any difficulties. Other labs at UCI have experience with RNA-seq; therefore, we do not anticipate major problems. RNA-seq has the advantage of being able to identify potentially important non-coding transcripts such as miRNAs and antisense transcripts (69-72).