Influence of Maternal Obesity, Diet and Exercise on Epigenetic Regulation of Adipocytes

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1. Epigenetics Mechanisms of Obesity: An Overview

1.1. Obesity

The prevalence of obesity and metabolic syndrome has been increasing at an alarming rate in children and in adults (Apovian, 2016; Balkau et al., 2002; Skinner and Skelton, 2014; Wilson and Grundy, 2003). Obesity is associated with increased risk for development of metabolic syndrome and chronic diseases (Andersen et al., 2016). Obesity is a leading cause of preventable death (Ofei, 2005) so there is an urgent need for understanding its causes in order to develop preventable strategies and treatment. One emerging potentially important contributing factor is epigenetic alterations, in effect heritable changes in gene expression, without changes in genotype, that increase the risk of obesity in offspring and perpetuate obesity across generations (Choi et al., 2013; Choi and Friso, 2010; Claycombe et al., 2015a; Claycombe et al., 2015b). Much progress has been made in identifying risk loci for obesity, specifically with respect to increased body mass index (BMI) or body adiposity (Locke et al., 2015; Speliotes et al., 2010). Epigenetic modification of genes acquired early in life by exposure to environmental factors, may contribute to the later development of obesity in offspring (Vogt et al., 2014), correlate with offspring adiposity (Dick et al., 2014), and are important for transgenerational propagation of changes in energy metabolism that promote weight gain and obesity (Desai et al., 2015b). Unlike alterations in genetic codes, epigenetic changes are modifiable by dietary energy and factors (Dong et al., 2014; Myzak et al., 2006; Myzak et al., 2007; Rajendran et al., 2011; Vares et al., 2014). This review focuses on the current understanding of the role of epigenetic mechanisms and alterations that regulate metabolic processes in beige and brown adipocytes, and how these epigenetic alterations might be transmitted to subsequent generations.

1.2. Epigenetics

The term 'epigenetics' can be defined as heritable changes in gene expression that are independent from changes in DNA such as mutations. Epigenetic alterations produce changes in phenotype without changes in genotype. Epigenetics broadly encompasses all those mechanisms whereby different cell types can be generated from the same progenitor cell. All of the cells that make up an individual contain the same complement of DNA, and hence genes, yet those cells express different sets of genes resulting in a phenotype. Epigenetic mechanisms such as DNA methylation and hydroxymethylation, chromatin structure (including histone modifications and nucleosome positioning), and non-coding RNA based mechanisms, occupy the interface between transcription factors/RNA polymerase, and DNA, and therefore are key to selective interpretation of the genome. Furthermore, these mechanisms seldom act independently, but occur in tandem, and can influence each other. For example, the non-coding RNA Xist can coat the copy of the X-chromosome from which it is derived, and recruit histone-modifying complexes such as the polycomb repressive complex, PRC2 (Lee et al., 1996; Lee, 2012, 2012). In turn, PRC2 methylates histone H3 on lysine 27, which sets the stage for DNA methylation and silencing of the X- chromosome (Lee, 2012).

1.2.1. DNA Methylation.

DNA methylation in mammals involves the addition of a methyl group to the 5' position of the cytosine of a CG base pair, often considered the "5th base" of DNA. While it does not hinder C-G bonding, the methyl group can prevent binding of factors to DNA, as it sticks out into the major groove of the DNA double helix. The enzyme DNMT1, which has a preference for hemi-methylated DNA, (Cheng,Blumenthal, 2008; Bestor, 2000; Jurkowska et al., 2011), is the maintenance methyltransferase that takes advantage of the palindromic CG base pairs to

methyrate the newly synthesized strand of DNA during replication. Enzymes DNMT3a and DNMT3b do not distinguish between unmethylated and hemi-methylated DNA, and therefore have traditionally been thought of as *de novo* methyltransferases that methylate DNA during early development (Cheng, Blumenthal, 2008; Bestor, 2000; Jurkowska et al., 2011). However, recent evidence suggests that DNMT1, 3a and 3b might share roles, and are not distinct in their mechanisms of action (Jeltsch, Jurkowska, 2014). DNMT2, initially classed as a DNA methyltransferase, was discovered to be a tRNA methyltransferase (Goll et al., 2006; Jeltsch, 2006; Rai et al., 2007; Jurkowski et al., 2008), primarily serving to methylate RNA. DNMT3L, which has an important role in early development, is not thought to function as a DNA methyltransferase, but instead helps stimulate *de novo* methylation by DNMT3A, and for the establishment of maternal genomic imprints (Jeltsch, 2006; Uysal et al., 2015). DNA methylation (5mC) typically occurs at about ~1% of the total genomic DNA in human somatic cells, or at about 70-80% of CpG sites (Ehrlich et al., 1982), yet is of great importance in proper maintenance of the genome. Indeed, mice that carry deletions for methylation enzymes demonstrate embryonic (Dnmt1 and Dnmt3b) or postnatal (Dnmt3a) lethality, underscoring their essential roles in development (Li et al., 1992; Okano et al., 1999; Bestor, 2000). All DNMTs use S-adenosylmethionine (SAM) as the methyl group donor, and S-adenosylhomocysteine (SAH), which is a product inhibitor of methyltransferases. Therefore, dietary changes such as increasing intake of folate or other sources of methionine that affect SAM or SAH concentrations in the body, have the ability to alter methylation patterns of DNA and other genomic histones (Crider et al., 2012; Takumi et al., 2015). More recently, the discovery of a novel modification of DNA, namely hydroxymethylcytosine, also termed the “6th base” of DNA, has spurred interest in the function of this modification (Tahiliani et al., 2009; Pastor et al., 2013).

1.2.2. Chromatin and chromatin remodeling.

Chromatin is essentially DNA complexed with histone and other non-histone proteins, that allows the cell to carefully package its 2 meter long strand of DNA into a 5-10 micron sized nucleus, a compaction of over 10,000 fold. At its most basic level, the fundamental repeating unit of chromatin is the nucleosome, which consists of 1.65 helical turns of 147bp of DNA wrapped around a core of eight histone proteins; 2 each of histones H2A, H2B, H3 and H4 (Luger et al., 1997). Additional levels of compaction exist, resulting in the structures we know as chromosomes. While compaction is a highly efficient means of storing DNA, it poses a challenge for cellular processes such as DNA replication or transcription. To contend with tightly packed chromatin, the cell has evolved molecular machines in the form of multiprotein complexes known as chromatin remodelers.

There are two main types of remodelers, namely histone modifying enzymes and ATP-dependent chromatin remodelers (Swygert, Peterson, 2014). The histone modifying enzymes are either 'writers' of the histone code, such as histone acetyltransferases or HATs, or 'erasers' such as histone deacetylases, or HDACs. Besides acetylation, several other chromatin modifications (Strahl, Allis, 2000; Gardner et al., 2011) have been discovered, including methylation, phosphorylation, ubiquitylation, sumoylation, crotonylation, and more recently, palmitoylation. All of these modifications can be influenced by diet. For instance, production of butyrate in the gut microbes influences histone acetylation, as butyrate is a histone deacetylase inhibitor (Donohoe et al., 2012). Other dietary molecules such as water-soluble B vitamins like biotin, niacin, and pantothenic acid can influence histone modifications (Oommen et al., 2005). Compounds in food such as genistein, found in soy products, affects both DNA and histone methyltransferases, often causing unwanted effects (Daniel, Tollefsbol, 2015;

Greathouse et al., 2012; Dagdemir et al., 2013; Xie et al., 2014; Vahid et al., 2015). If these and other food factors are actively ingested during pregnancy, and alter epigenetic enzyme activity (Vahid et al., 2015), they can produce gene expression changes in the progeny that last for many generations.

The other type of chromatin modifying enzymes, the ATP dependent chromatin remodelers, influence the physical positioning of nucleosomes on DNA, thereby facilitating or hindering transcription factor binding to cognate sites via rotational positioning of DNA. ATP dependent chromatin remodelers are divided into 4 main families [switch mating type/sucrose nonfermenting (SWI/SNF), imitation switch (ISWI), chromodomain helicase DNA binding (CHD), and Inositol 80 (INO80) complexes], and the protein complexes that make up these families are crucial for the assembly of chromatin structures leading to gene expression, replication and other biological functions (Smith, Peterson, 2005; Swygert, Peterson, 2014). Direct evidence for chromatin remodeler function in obesity is evident from whole body disruption of the BRD2 protein, which has SWI/SNF-like chromatin remodeler functions and results in lifelong severe obesity in mice (Wang et al., 2013; Sun et al., 2015). These studies underscore the importance that ATP-dependent chromatin remodeling mechanisms play in the cross-generational prevention or perpetuation of obesity.

1.2.3 Non-coding RNAs.

The third type of epigenetic mechanism is mediated by non-coding RNAs, which are not translated into protein, but instead influence gene expression. Several types of noncoding RNAs exist (Lee, 2012), including microRNAs (21-24nt pieces of RNA), which repress transcription and translation; long non-coding RNAs such as lincRNAs, which serve to recruit transcriptional complexes, and circRNAs, which can 'sponge' up microRNAs in the cell,

providing a rapid means of regulating gene transcription (Danan et al., 2012; Hansen et al., 2013; Memczak et al., 2013). Some non-coding RNA changes in response to the diet (Lillycrop, Burdge, 2015; Jimenez-Chillaron et al., 2012; Soubry et al., 2014) include those implicated in obesity risk (Martinez et al., 2012; Milagro et al., 2013). Several miRNAs that either promote (e.g., miR-193b-365 and miR-196a) or inhibit (miR-155 and miR-27) brown/beige adipogenesis have been identified (Xu et al., 2015). To date, there have been no studies on dietary influences of lincRNAs and circRNAs or their role in obesity. These are sure to be of future areas of interest. The recent discovery that exosomes carry non-coding RNAs including miRNAs has huge implications for understanding influences of maternal diet on obesity (Ferrante et al., 2015; Zhang et al., 2016). For instance, maternal diet could cause changes in exosomal miRNAs, which can then be transferred to infants via breast milk (Fooladi et al., 2013).

Thus, epigenetic mechanisms are responsive to environmental stimuli, including diet, and these changes can be propagated for several subsequent cell divisions in the case of mitotic changes and even generations in the case of meiotic changes. An important unanswered question is how stable are epigenetic changes to subsequent environmental stimuli, and, can they be reversed by changing the diet or physical activity. Understanding the players and basic mechanisms of epigenetic changes resulting from maternal and perhaps paternal dietary intake could make major contributions towards understanding therapeutic targets to prevent occurrence of obesity, and to lower the risk of developing metabolic syndrome and other obesity-related diseases in later life.

1.3 Parental high-fat diet and obesity affect trans-generational epigenetic modifications

Pre-pregnancy maternal BMI is a strong predictor of a child's BMI (Schou Andersen et

al., 2012). Parental obesity is a primary risk factor for child obesity and for child obesity tracking into adulthood (Whitaker et al., 1998). Parents influence a child's BMI/adiposity trajectory through biological (e.g., genetics, epigenetics) mechanisms as focused upon in this review, but also through their behaviors (e.g. modeling, praising, and reinforcing healthy or unhealthy eating and physical activity behaviors), and exposing the child to modifiable environmental factors (e.g. sedentary behaviors, energy dense foods) (Eriksson et al., 2003; Sonnevile et al., 2012; Swinburn and Egger, 2004; Wrotniak et al., 2004). These mechanisms can also interact. For instance, a maternal energy dense diet could produce epigenetic alterations that increase obesity risk of offspring through phenotypic changes in metabolism and/or preference that is manifested as behavior, such as increasing the child's motivation to eat energy dense foods.

Indeed, maternal energy surfeits due to consumption of high-fat (HF) diets increase risk for obesity in offspring (Benkalfat et al., 2011; Caluwaerts et al., 2007; Franco et al., 2012), perhaps through epigenetic modifications transmitted from parent to offspring. Feeding an energy dense, high-fat diet to Japanese Macaques alters fetal chromatin that increases access of transcription factors to target DNA binding sites (Aagaard-Tillery et al., 2008). Maternal high-fat feeding can also alter DNA methylation of other metabolically important organs. A high-fat diet (45% kcal from fat) reduces the expression of hepatocyte growth regulator gene *Cdkn1a*, leading to reduced liver cell size (Dudley et al., 2011), suggesting detrimental, long-term consequences for the offspring.

Obesity in childhood is associated with early expression of hypertension (McGill, 1997), an atherogenic lipid profile (Davis et al., 2001) and type 2 diabetes mellitus (Steinberger et al., 2001). Whether a maternal HF diet directly influences epigenetic pathways to cause obesity and early expression of metabolic dysfunction in offspring has been mainly addressed in

animal models. In a mouse model of diet-induced obesity, feeding dams a HF (62% fat) diet during gestation resulted in female offspring with an obese phenotype, hypertension, glucose intolerance, hypertriglyceridemia, as well as decreased serum adiponectin and increased leptin concentrations (Masuyama et al., 2015). In the same study, *adiponectin* gene expression in visceral fat was decreased due to an increased amount of the dimethylated form of histone 3 lysine 9 (H3K9) protein found in the promoter regions of the *adiponectin* gene. Leptin gene expression was associated with increased monomethylated form of histone 4 Lysine 20 (H4K20) protein found in the promoter regions of the *leptin* genes in the same adipose tissue depot (Masuyama et al., 2015). All of these observed phenotypes and epigenetic changes were abolished with a normal fat (NF; 12% fat) maternal diet across 3 generations, providing evidence that a lower fat content in the diet can help reduce obesity-associated metabolic dysfunction by reversing previous maternal HF diet-induced epigenetic modification.

Maternal HF diet (39% fat compare to NF diet of 12% fat) effects on body weight changes and body length via DNA methylation changes has been examined over 3 generations of hybrid mice (e.g. C57Bl/6:129) (Dunn and Bale, 2009). Generation-dependent differences in body weight increases were found in that the difference was present in the first, but not in the second generation female and male offspring. Dunn et al. (Dunn and Bale, 2009) found that the progeny of high-fat fed dams had greater body weights and elevated fasting concentrations of glucose and leptin, with increased insulin resistance due to differential DNA methylation of GH axis modulators including IGF-1 and GHSR genes (Dunn and Bale, 2009). The same study also showed that mRNA levels of genes involved in linear growth (equivalent of height in human) such as growth hormone secretagogue receptor (GHSR) in the hypothalamic regions of the brain, and associated changes in GHSR promoter DNA methylation levels were sex-dependent. For example GHSR promoter DNA methylation was

decreased at +72 bp unstream of transcription start site location in male F2 offspring while DNA methylation were decreased at -31 bp downstream of the transcription start site location in the female. Moreover, mRNA levels of the transcription factor that binds to GHSR promoter (e.g. GHSR transcriptional repressor AF5q31) decreased in female, but not in male brains (Dunn and Bale, 2009). These finding indicate that parental HF diet effects are differentially transmitted and inherited via generation- and gender-dependent pathways.

In addition to a maternal HF diet, a paternal HF diet and obesity also produce transgenerational effects on offspring obesity. In a recent study (Fullston et al., 2013), male C57BL6 mice were fed either a HF (40% fat) or control (CD; 21% fat) diet for 10 weeks. Interestingly, a paternal HF diet induced earlier and greater increases in adipose tissue in F1 female compared to male mice. Furthermore, insulin resistance and increased adiposity occurred in F2 female, but not F2 male, mice. Although paternal diet did not affect male offspring adiposity and insulin resistance, a paternal HF diet altered the expression of 414 mRNAs and 11 microRNAs (miRNA) in testes and produced a 25% reduction in global methylation of sperm. These findings highlight the importance of the paternal diet on miRNA and methylation status of sperm and a paternal mechanism for epigenetic programming of offspring obesity.

While there are independent effects of maternal and paternal consumption of HF diet on epigenetic alterations and offspring obesity risk, whether maternal and paternal HF diets exert additive effects has not been widely studied. Two recent studies tested the effects of HF diets and epigenetics. The first employed a maternal HF diet across 3 generations of male offspring to test whether epigenetic pathway alterations contribute to additive liver lipid accumulation and hyperlipidemia-induced liver endoplasmic reticulum (ER) stress across generations (Li et al., 2012). The F2 generation male mice had obesity, increased hepatic lipogenesis and

steatosis, hepatic ER stress, and decreased histone 3 lysine 9 (H3K9) methylation compared to control. Furthermore, all outcomes showed additive effects over 3 generations. These findings indicate that HF diet exposure across generations of mothers (dams) induces additive epigenetic changes in H3K9 to induce obesity and hepatic steatosis and that the phenotypic health-related changes in male offspring increase in severity across generations. The second study (Huypens et al., 2016) tested whether epigenetic inheritance could increase the propensity to develop type-2 diabetes and obesity. They found that when both male and female parents are fed on a HFD, the female offspring were more obese relative to if neither or only one parent was fed a HFD, suggesting approximately equal contributions from male and female gametes. Interestingly, while male offspring from HFD-fed parents were heavier than normal diet fed parents, the difference in weight was not statistically significant, indicating that female progeny are more susceptible relative to males to develop obesity. However, this study did not test whether these effects were transgenerational. Based on these two studies, if the same is true for humans, the increase in maternal and paternal obesity and HF diets that has now occurred across multiple human generations could have important implications for ever greater risk of epigenetic transmission of obesity and disease.

1.4 Exercise-induced epigenetic alterations

Aerobic exercise and physical activity are effective for the prevention and treatment of many diseases (Vina et al., 2012) and one mechanism for these health effects are through exercise-induced epigenetic alterations that reduce risks of obesity and disease. The activity of many of the enzymes discussed above that control DNA methylation and histone post-translational modifications are likely altered by changes in concentrations of metabolites during and after exercise (Pareja-Galeano et al., 2014).

A beneficial response to aerobic exercise training is the ability to regenerate ATP through aerobic metabolism. The effects of endurance exercise have traditionally focused on skeletal with reliable increases in mitochondrial enzyme activity and the number and size of mitochondria that increases oxygen consumption (Holloszy and Coyle, 1984). The resulting reduction in intracellular oxygen concentration during exercise may be a key stimulus that promotes epigenetic changes within skeletal muscle (Pareja-Galeano et al., 2014).) Aerobic exercise may also protect against HF diet-induced epigenetic alterations that downregulate metabolic master regulator, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) in skeletal muscle with the beneficial result of delaying the onset of type 2 diabetes (Santos et al., 2014).

Recent evidence demonstrates that exercise training effects also occur within adipose tissue. In rats, voluntary running increased WAT mitochondrial number and protein content (e.g. cytochrome c, COXIV-subunit I, and citrate synthase activity) (Laye et al., 2009). Aerobic exercise also increased mRNA concentrations of mitochondria synthesis regulators including PGC1-alpha, PPAR-delta, and NRF-1 in skeletal muscle and BAT (Seebacher and Glanville, 2010). These results are tempered by a human trial that found 6 months of exercise produced no changes in the cellular composition of adipose tissue based on mRNA expression of markers of adipocytes, preadipocytes, brown adipocytes, and macrophages (Ronn et al., 2013). However, the trial did find an increase in adipose tissue DNA methylation after 6 months of exercise. Within adipose tissue, 21 candidate genes for type 2 diabetes and 18 for obesity had at least one CpG site with altered DNA methylation. Changes in mRNA expression were observed in 6 of these genes. Ten of the CpG sites were within the KCNQ1 gene body and 6 within TCF7L2 and both genes have functional roles in type 2 diabetes risk (Travers et al., 2013). Two genes (HDAC4, NCOR2) found to have increased DNA methylation and

decreased mRNA expression in adipose tissue after exercise training were silenced in 3T3-L1 adipocytes. Silencing of either HDAC4 (histone deacetylase that quenches GLUT4 transcription in adipocytes (Weems et al., 2012) or NCOR2 (nuclear co-repressor that regulates lipid metabolism (Watson et al., 2012) resulted in increased lipogenesis in basal and insulin stimulated states (Ronn et al., 2013). Exercise training appears to affect adipocyte DNA methylation to affect adipocyte metabolism, including lipogenesis.

2. Factors influencing adipogenesis: current status of knowledge

2.1 Stages of adipogenesis and adipocyte differentiation

Enlargement of existing fat cells (hypertrophy), increased proliferation (hyperplasia), and an increased rate of differentiation of adipocytes from their precursor cells (Cushman et al., 1981; van Harmelen et al., 2003) can all contribute to an increase in adipose tissue mass, and consequently obesity. Adipogenic processes are suggested to be mediated by changes in adipogenic gene promoter methylation pattern thereby increasing gene expression.

Adipogenesis is initiated by activation of peroxisome proliferator-activated receptor γ (PPAR γ), followed by binding of CAAT-enhancer binding protein β (C/EBP β) to the PPAR γ promoter (Rosen and Spiegelman, 2001), which leads to activation and binding of C/EBP α and terminal differentiation of adipocytes (Farmer, 2006). Studies have also suggested a role for histone modifications in adipogenic gene transcription. For example, PAX transactivation domain-interacting protein (PTIP), a protein known to associate with transcriptionally active chromatin by interacting with histone 3 lysine 4 (H3K4) methyltransferases, upregulates PPAR γ and C/EBP α expression during adipogenesis (Cho et al., 2009).

It is important to note that not all epigenetic modifications of metabolic genes in adipose tissue contribute to adipogenesis. For example, both mesoderm-specific transcript (Mest)

(Lefebvre et al., 1998; Nishita et al., 1999), which is upregulated in white adipose tissue of obese mice fed dietary high fat (Takahashi et al., 2005). However, expression of Mest was positively associated with energy intake, while its CpG methylation pattern was unaffected (Koza et al., 2009). Furthermore, promoters of undifferentiated adipocyte stem cells are generally hypermethylated until these cells are induced to differentiate (Sorensen et al., 2010). However, differentiation does not seem to trigger any notable specific changes in DNA methylation pattern or alteration in adipogenic gene expression (Koch et al., 2013; Noer et al., 2007; Noer et al., 2006; Sorensen et al., 2010). Recent studies have shown that miRNAs play important roles in the development of obesity by regulating adipocyte differentiation (Kloting et al., 2009; Li et al., 2013). Of the large number of miRNAs expressed in adipose tissue (Kloting et al., 2009), only a few have been implicated in modulating relevant metabolic functions as glucose tolerance regulation (miR-145) (Kloting et al., 2009) and lipogenesis (miR-181a) (Choi et al., 2013). Although these miRNAs are of endogenous origin, nutrients and other bioactive food components have been reported to modulate their expression (Ross and Davis, 2011).

Maternal diet can affect fetal growth. Chen et al (Chen et al., 2012) found that maternal consumption of a diet high in both fat (30%) and sugar (36%) induced maternal obesity while reducing placental growth, nutrient transfer and fetal growth. This diet also increased placental fatty acid transporter protein and placental growth regulatory imprinted genes including that for insulin-like growth factor-2 (IGF2) (Chen et al., 2012).

Major types of adipocytes and energy metabolism regulation. Of the two major types of adipose, white adipose tissue (WAT) and white adipocytes are mostly involved in storage of energy in the form of triacylglycerides; whereas brown adipose tissue (BAT) and brown

adipocytes are relatively rich in mitochondria with the primary function of oxidizing lipids to generate heat (Meyer et al., 2010). Mitochondria-rich brown adipocytes and functionally active BAT tissue are found in the human (Cypess et al., 2009; Muzik et al., 2013; Ouellet et al., 2012; Xu et al., 2011) making BAT a potentially important anti-obesogenic tissue. The contribution of BAT to whole body energy expenditure is 20% at maximal rate (Rothwell and Stock, 1983). Beige adipocytes also arise from resident precursor stem cells in adipose tissue, and differentiate into adipocytes with an increased number of mitochondria, turning the cell into a 'more brown like or beige' color adipocyte (Wang et al., 2013b). Beige adipocytes have intermediate levels of mitochondria and uncoupling protein-1 (UCP-1) expression (Virtanen et al., 2009) compared to brown adipocytes, and arise from white adipocyte precursor Myf5-negative cells (Seale et al., 2008) activated by PRDM16 (Seale et al., 2008).

Adipocyte cellular metabolism regulation. During pregnancy, maternal diet can influence mitochondrial development in a manner that produces heritable alterations in mitochondrial function (Ding et al., 2010; Reusens et al., 2011). Recent studies indicate that epigenetic modification of cytosine in mtDNA is much more common than previously believed, and promoter hypermethylation is correlated with mitochondrial DNA (mtDNA) alteration and nuclear DNA crosstalk (Xie et al., 2007). Because mitochondria have a central role in regulating cellular energy metabolism, alterations in their development, particularly in tissues important in regulating energy balance, can be expected to predispose offspring to increased risk for obesity. Both the quantity and quality of mtDNA can be influenced by protein intake (Jia et al., 2013; Park et al., 2003). Offspring of dams fed a low-protein diet during gestation and lactation have lower concentrations of mtDNA in liver and muscle, an effect not reversed by feeding adequate protein after weaning (Park et al., 2003). Similarly, offspring of rats fed a

high-fat diet during gestation and lactation have decreased mtDNA copy number and expression of mitochondrial encoded subunits of Cytochrome C Oxidase mRNA in both liver and kidney (Taylor et al., 2005). The methylation of genes involved in mitochondria function, such as PPAR- γ coactivator-1 α (PGC- α), are negatively correlated with PGC- α mRNA and mtDNA in type 2 diabetic patients (Barres et al., 2009). Taken together these effects were associated with subsequent development of insulin resistance and pancreatic beta-cell dysfunction (Taylor et al., 2005).

2.2. Fetal growth factors that influence adipocyte biology

Studies have suggested that body fat percentage in later life is programmed *in utero* (Elia et al., 2007). Currently, little is known about how maternal obesity, maternal or offspring diet, or maternal or offspring exercise can modify epigenetic regulation of beige and brown adipocyte differentiation. We have shown that high-fat diet fed rat offspring from mothers that consumed a low-protein diet through pregnancy and lactation have reduced induction of beige adipocytes in subcutaneous adipose tissue via reduction in fibroblastic factor 21 (FGF21) and increased Histone H3 Lysine 9 (H3K9) Methyltransferase G9a expression (Claycombe, 2016). Findings from this study begin to elucidate that one mechanism by which maternal protein malnutrition causes offspring to have low energy expenditure that increased vulnerability to obesity and metabolic alterations when exposed to high dietary fat postnatal environment is via decreased beige adipocyte number.

Both maternal high-fat and low-protein diets result in abnormal (both low and high) birth weight of offspring, thereby predisposing them to obesity (Buckley et al., 2005; Choi et al., 2013; Curhan et al., 1996a; Curhan et al., 1996b; Roseboom et al., 1999; Whitaker and Dietz, 1998; Whitaker et al., 1998). During critical periods of fetal development, sustained exposure

to fetal growth factors (e.g. insulin like growth factor 1 (IGF1) and IGF2) may program adipose tissue of offspring for obesity. IGF2, one of the best characterized epigenetically imprinted genes, is associated with greater body weight (Gaunt et al., 2001; Souren et al., 2009) and obesity (Faienza et al., 2010; Perkins et al., 2012; Roth et al., 2002). IGF2 DNA methylation and IGF2 gene expression increase with increased subcutaneous fat mass in human (Huang et al., 2012). We and others have demonstrated that the maternal diet (both low protein or HF) results in rapid adipose tissue growth in rat offspring, resulting in obesity and both increased IGF2 gene expression and DNA methylation (Choi et al., 2013; Sferruzzi-Perri et al., 2013). We also demonstrated that a postnatal HF diet alone increases IGF2 gene expression and DNA methylation (Choi et al., 2013). IGF1 induces adipocyte differentiation (Scavo et al., 2004) and stimulates metabolic hormone secretion (Maillard et al., 2011). Highly mitochondrial dense and metabolically active BAT is an IGF-I target tissue and fetal BAT has an especially high number of IGF1 receptors (Lorenzo et al., 1993; Valverde et al., 2004).

Leptin is another metabolic hormone that plays a role in epigenetic programming of adipocytes. The major function of leptin is to regulate food intake and energy expenditure by working through hypothalamic signaling pathways (Campfield et al., 1995; Halaas et al., 1995; Pellemounter et al., 1995). Milagro et al. (Milagro et al., 2009) showed that expression of the leptin gene can be affected by high-fat feeding and by leptin gene promoter methylation in rat adipocytes; increases in adipose tissue mass were inversely associated with leptin promoter methylation. Further, CpG islands in the leptin gene promoter are hypermethylated in preadipocytes, but become demethylated, and, as a consequence, less active in differentiated 3T3-L1 adipocytes (Yokomori et al., 2002) and human adipocytes (Melzner et al., 2002). Treatment of preadipocytes with leptin reduces adipogenic transcription factor mRNA expression as well as adipocyte differentiation (Rhee et al., 2008). Taken together, these

findings suggest that demethylation during adipocyte differentiation contributes to increased expression of the leptin gene (Yokomori et al., 2002), which then serves as a feedback inhibitor to reduce further adipogenesis.

2.3. Transcriptional regulation of adipogenesis and adipocyte differentiation

Transcription factors are proteins that regulate gene expression by integrating signals from the environment and epigenetic events. Several transcription factors are involved in adipogenesis and differentiation of adipocytes, and dysregulation of many of these are thought to play a role in development of obesity. Brown, beige and white adipocytes have distinct structures and functions, and this uniqueness is due to differential expression of transcription factors within these three types of fat. Interestingly, a number of developmental transcription factors that play roles in embryonic development are strongly differentially expressed between white adipose tissue in different regions of the body, as well as between brown and white adipose tissue. More recently, transcription factors involved in the epithelial to mesenchymal transition (EMT), a phenotypic cell state switch in early development, have been implicated in adipogenesis. As transcription factors interact with the epigenetic machinery of the cell, switching critical transcription factor networks on or off can have drastic effects on cell phenotype and in adipogenesis, resulting in obesity. However, as epigenetic changes are potentially reversible, this also means that we can reverse these effects by targeting the epigenetic changes responsible. Here, we will briefly highlight some of the major transcription factors that have been identified to play important roles in obesity via epigenetic regulation.

2.3.1 PPAR γ and C/EBP. The nuclear factor peroxisome proliferator-activated receptor (PPAR γ), is a master regulator of adipocyte biology, and is the cellular target of many anti-

diabetic thiazolidinedione drugs. PPAR γ is necessary (Rosen et al., 2002) for the differentiation of mouse fibroblasts into adipocytes. Critical roles for PPAR γ in adipocyte development and function, such as genes involved in lipid synthesis, storage and metabolism, secretion of adipokines, and sensitivity to insulin have been reported in the literature, following studies on animal models as well as existing human mutation analyses (Rangwala and Lazar, 2000; Rangwala et al., 2003; Rangwala and Lazar, 2004; Gray et al., 2005; Lehrke and Lazar, 2005). Functionally, PPAR γ binds to peroxisome proliferator response elements (PPREs) in DNA as a heterodimer with RXR α in a head-to-tail orientation (Gearing et al., 1993; IJpenberg et al., 1997). Interestingly, ChIP analysis confirmed colocalization of C/EBP α at the majority (~91%) of PPAR γ -binding regions (Lefterova et al., 2008). A study using mouse fibroblasts derived from a PPAR γ conditional null mouse showed that while C/EBP α by itself did not induce adipogenesis, rather it was able to drive adipogenesis initiated by re-introduction of PPAR γ in the cells (Rosen et al., 2002).

PPAR γ binding can be influenced by dietary intake, as some studies indicate expression of PPAR γ and its downstream targets are altered by dietary compounds including apple polyphenols, dietary fish oils and maternal diet (Boque et al., 2013; Li et al., 2014; Desai et al., 2015a). While it is reasonable to predict that PPAR γ binding was altered under these conditions, few of these studies confirmed direct binding events by PPAR γ in response to these dietary stimuli. Therefore, *in vivo* PPAR γ ChIP-sequencing studies are warranted to understand more fully the role of maternal diet in altering PPAR γ DNA binding events, leading to epigenetic reprogramming.

2.3.2 EBFs in adipogenesis and obesity.

Although most studies have focused on the role of PPAR γ and C/EBP proteins, a large number of other transcription factors are regulated

during adipogenesis. One such group of factors, the Early B-cell Factor (Ebf) (O/E) family of helix-loop-helix transcription factors has three members, Ebf1, 2, and 3, which are all expressed in adipocytes, and have adipogenic potential in multiple cellular models. EBF proteins also interact with PPAR γ and C/EBP proteins: C/EBP β and δ are among the earliest factors to respond to adipogenic stimuli in a committed precursor cell, and these in turn induce expression of PPAR γ and C/EBP α (Jimenez et al., 2007).

Extending these findings, Seale and colleagues (Rajakumari et al., 2013) used genome-wide ChIP-sequencing approaches to find that Ebf2 regulated brown versus white adipocyte identity by specifically recruiting PPAR γ to brown adipocyte-specific PPAR γ gene targets. When Ebf2, which is highly expressed in brown fat cells (but not beige) relative to white adipocytes, was expressed at greater levels in white adipocyte cells, it was redirected PPAR γ to brown fat target genes, thereby reprogramming the fate of the white cells (Rajakumari et al., 2013). Simply expressing Ebf2 in beige or white adipocytes was sufficient to trigger this reprogramming, and to increase thermogenic capabilities of these cells (Rajakumari et al., 2013), suggesting exciting possibilities for therapeutics.

EBF proteins directly bind to the consensus DNA sequence 5'-CCCNNGGG-3' as homo- or heterodimers (Hagman et al., 1995), and mediate transcriptional activation and repression by interaction with p300 histone acetyltransferase (Zhao et al., 2003), and in conjunction with the Zfp423/Zfp521 protein (Harder et al., 2013), interacts with the Mi-2/NuRD chromatin remodeling complex. While the upstream signaling events leading to Ebf upregulation in brown fat cells is unknown, the fact that epigenetics plays an important role in their mode of action is encouraging for therapeutic reasons.

2.3.3 The FOX family.

Forkhead box (FOX) proteins are a family of around 40

transcriptional regulatory proteins that control diverse cellular processes including differentiation, metabolism, development, proliferation, and apoptosis (Friedman,Kaestner, 2006; Myatt,Lam, 2007; Wolfrum et al., 2003; Arden, 2008). Fox family members act as 'pioneer transcription factors' and serve to alter chromatin structure to allow other proteins to bind (Friedman,Kaestner, 2006; Myatt,Lam, 2007). The three predominant members (Foxo1, Foxo3 and Foxo4) of the mammalian FOXO subfamily regulate a wide variety of cellular processes both during development and in the adult. The FOXO1 protein can increase resistance to insulin, by upregulating insulin synthesis, and by suppressing insulin sensitivity in the liver, brown and white adipose tissue. Additionally, FOXO1 also suppresses adipogenesis and adipocyte size, thus decreasing energy expenditure. Environmental cues can regulate FoxO protein cellular localization by direct signaling via either the JNK, AKT or related pathways. For instance, black tea polyphenols mimic insulin/IGF-1 signaling by inducing rapid FOXO1a phosphorylation, thereby excluding it from the nucleus and preventing glucose production (Cameron et al., 2008). Diets containing docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, reduced the expression of FoxO1 and FoxO3 in the liver and adipose tissue of pigs, suggesting a role of FoxO factors in helping to ameliorate obesity. In support of this idea, FoxO1 haploinsufficient mice showed increased PPAR γ gene expression in adipose tissue, and increased insulin sensitivity.

2.3.4 The SNAIL family (SNAIL, SLUG) and other EMT proteins (ZEB1, TWIST).

Besides GATA factors, studies have recently started to reveal novel roles of EMT transcription factors in adipogenesis and adipocyte lineage commitment. The EMT, or epithelial to mesenchymal transcription, describes the series of gene expression changes that result in conversion of an epithelial attached cell that becomes phenotypically elongated, migratory, and

loses apico-basal polarity. Many regulatory transcription factors play important roles in this process, including Snail, Slug, Zeb1, Zeb2 and Twist proteins.

The first study to implicate EMT regulators in adipogenesis demonstrated increased *SLUG* expression in white adipose tissue in humans (Perez-Mancera et al., 2007). This study also demonstrated that Slug-deficient mice had reduced white adipose tissue, while the converse, increased white adipose tissue mass, was noted in Slug-overexpressing mice (Perez-Mancera et al., 2007). Another study suggested that there were higher mRNA levels of Slug (and Snail) in subcutaneous adipose tissue taken from obese subjects relative to their lean counterparts (Bourlier et al., 2012).

The transcription factor Snail regulates adipocyte differentiation, both by repression of PPAR γ expression (Lee et al., 2013), and inhibition of adiponectin expression (Park et al., 2012). Both these studies demonstrated that repression of these two genes occurs via Snail binding to the E-box sequences in the gene promoters, and (presumably) by interaction with chromatin regulatory proteins such as HDACs and G9a to repress gene expression, although this was not demonstrated in these studies. Overall, these results point to the role of Snail as an inhibitor of differentiation, and necessary to maintain a 'stem cell'-like phenotype, as observed by groups working on cancer and stem cells (Battula et al., 2010; Dang et al., 2011; Lin et al., 2014; Zhou et al., 2014; Horvay et al., 2015). Indeed, bone-marrow derived stem cells that are depleted for Snail show a premature differentiation into osteoblasts or adipocytes (Batlle et al., 2013).

Another EMT protein, Zeb1, was implicated in adiposity when GWAS studies linked an "obesity" gene to human chromosome 10p11–12, which contains Zeb1. A recent report linked a ZEB1 gain-of-function mutation to increased adiposity (Kurima et al., 2011), which also fits the hypothesis that Zeb1 is a positive regulator of adipogenesis. Consistent with this idea, a recent

large-scale transcription factor overexpression screen identified several novel positive regulators of adipogenesis in 3T3L1 cells. This screen identified Zeb1 as essential for adipogenesis (Gubelmann et al., 2014). The authors further demonstrated that Zeb1 genomic localization is highly correlated with known adipogenic regulators such as C/EBP β (Gubelmann et al., 2014), suggesting that Zeb1 is needed to regulate expression of transcription factors during early adipogenesis.

Expression of another key EMT-related transcription factor, Twist 1, has been noted in primary osteoblastic cells derived from newborn mouse calvariae (Murray et al., 1992), and in mouse brown and white adipocytes (Pan et al., 2009; Pettersson et al., 2010, 2011). The mechanism of action of Twist1 in differentiation of 3T3L1 preadipocytes appears to be similar to that of Snail and Slug, in that it regulated expression of PPAR γ during Day 4 of differentiation. However unlike Snail and Slug, knockdown of Twist1 did not impair lipid formation (Ma et al., 2014), suggesting a less critical role in differentiation. In contrast to Zeb1 heterozygous mice, Twist1 heterozygous mice were resistant to obesity due to altered mitochondrial metabolism in brown fat cells (Pan et al., 2009).

Finally, a very recent study showed a significant role for the KRAB-zinc-finger transcription factor Trim28 in regulating body weight (Dalgaard et al., 2016). The authors noted that genetically identical (syngenic) mice bearing a heterozygous Trim28 protein were able to randomly switch their phenotype from a normal body weight to being obese. While genetically identical in all other aspects, the mice seemed to respond to something in their environment and form two distinct groups, the obese-on and obese-off phenotypes, and once established, also remained stable over generations, suggesting epigenetic mechanisms are at play. These experiments suggested that environmental cues from hormonal or dietary changes can be

“sensed” by chromatin-based mechanisms, thereby driving morphological changes to adapt to the environment.

3. **Preventive strategies: Drugs targeting epigenetic modifications: a ‘cure’ for obesity?**

Therapeutic agents that inhibit epigenetic modifications such as histone acetylation and DNA methylation have long been used in the cancer field to treat patients with some efficacy in ameliorating these disease states. Therefore, ever since studies showed that these same modifications also play significant roles in obesity, it was to be expected that these same drugs would also be tested clinically in treatments targeting obesity and its related metabolic syndromes such as type 2 diabetes.

Clinical studies featuring the histone deacetylase inhibitors valproate and butyrate are currently being tested clinically. The use of valproic acid for treatment of obesity has yielded inconclusive results, with some groups claiming increased weight gain with chronic treatment (Belcastro et al., 2013), while others claiming just the opposite (Zuo et al., 2015). In a recent study (Avery and Bumpus, 2014), VPA was shown to activate AMP-activated protein kinase (AMPK), a key drug target in treating diabetes and obesity, suggesting a role for valproate in the treatment of type 2 diabetes.

Besides drugs that target histone modifications, miRNAs are now emerging as viable targets for therapy. For instance, a screening study of adipogenic miRNAs in human pre-adipocytes revealed that miR-143 effectively inhibited adipocyte differentiation and adipogenesis (Esau et al., 2004). The action of miR-143 during adipogenesis targets MAP kinase pathways (Esau et al., 2004). Further, overexpression of miR-143 in obese mice models inhibited insulin-stimulated AKT activation, and impaired glucose metabolism (Jordan et al., 2011). Taken together, these studies suggest that miR-143 is a strong candidate for

targeting obesity. Overexpression of miR-27a in 3T3-L1 pre-adipocytes suppressed PPAR γ expression and adipocyte differentiation, suggesting that miR-27a mimics can be used to regulate adipogenesis and differentiation of adipocytes (Kim et al., 2010). More recently, a role for miR-27 in brown fat metabolism has been implied, and miR-27 inhibition was suggested as a novel therapeutic approach to increase the beige/brown fat mass (Sun and Trajkovski, 2014). These studies therefore demonstrated that microRNAs could potentially be used as effective targets to combat adipogenesis and obesity.

Bioactive nutrients and role in reducing adiposity via epigenetic regulation.

Whether maternal or paternal diets supplemented with bioactive nutrients, termed constituents in foods which are responsible for changes in health status (Weaver, 2014), influence epigenetic pathways to regulate human and animal obese phenotypes remain to be determined. Few studies have demonstrated that bioactive nutrients regulate adipocyte metabolism and differentiation. Fisetin, a flavonoid found in many fruits and vegetables, inhibits PPAR γ acetylation *in vitro* (in 3T3-L1 cells) via Sirt1, while inhibition of Sirt1 activity induces PPAR γ acetylation and thus contributes to increased lipid accumulation and adipocyte differentiation (Mir et al., 2015). Another phenolic compound (including chlorogenic acid, phloridzin, quercetin, catechin, epicatechin, procyanidin, and rutin), found at high concentrations in fruits such as apples, regulates adipogenesis by differentially regulating DNA methylation. Rats fed an apple polyphenol supplemented diet (700 mg/kg body weight) showed slower body weight gain and fat deposition and improved glucose tolerance compared to high-fat sucrose fed rats (Boque et al., 2013). These phenotypes were associated with reduced leptin (adipocyte derived enhancer of energy expenditure) and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (Ppargc1 α) mRNA levels, and

differential methylation patterns of Leptin and Ppargc1 α promoters in adipocytes from apple-supplemented rats. Other bioactive phenolics, such as anthocyanins found in dark color fruits, modulate epigenetic pathways to reduce HF diet-induced inflammation (Benn et al., 2014) and cancer risk (Fimognari et al., 2008; Gerhauser, 2008; Wang et al., 2013a). However no studies have shown epigenetic modulations of anthocyanin on adipocyte differentiation and metabolism leading to obesity.

Role of methyl donor vitamins on adipogenesis and epigenetic modification.

Maternal folic acid or vitamin B12 restrictions regulate body composition and fat metabolism in animal studies. A recent study showed that offspring born to pregnant/lactating rats that were fed folate and vitamin B12 deficient diet had lower birth weight, higher visceral fat mass, dyslipidemia, and increased concentrations of systemic inflammatory cytokines compared to control group (Kumar et al., 2013). Furthermore, folic acid supplementation seems to have beneficial effect on fat oxidative metabolic pathway. In one study, folic acid supplementation in leptin receptor deficient genetically obese db/db mice showed improved lipolytic responses (Lam et al., 2009). Folate also influences CpG island methylation in adipogenic gene promoters such as PPAR γ and C/EBP α (Yu et al., 2014).

Role of maternal exercise on offspring adipogenesis and epigenetic modification

In humans, aerobic exercise improves maternal aerobic and muscular fitness and slows weight gain without compromising fetal growth (Clapp et al., 1992). Indeed, exercise during pregnancy may be advantageous, especially for overweight or obese pregnant women as it reduces the risk of delivering a large for gestational age (LGA) infant (Ferraro et al., 2012). Infants born LGA are at greater risk to be overweight throughout all stages of life (Ferraro et

al., 2012). Children of mothers who gain more than recommended amounts of weight during pregnancy have greater BMI, total and abdominal fat mass, and inflammatory markers. Aerobic exercise during pregnancy may reduce predisposing risk factors during fetal development and provide health benefits for the child later in life (Ferraro et al., 2012; Fraser et al., 2010). Controlled studies of the effects of maternal aerobic exercise in humans on epigenetic changes to placental and fetal DNA and placental and fetal mitochondrial function and metabolism within adipocytes have not yet been conducted. However, there is observational evidence that aerobic exercise during pregnancy in humans can reduce fetal size (Clapp and Capeless, 1990; Hopkins et al., 2010). More direct evidence of the effects of maternal aerobic exercise on birth size is available from rodent models. Maternal exercise reduces offspring birth weight and in adulthood protects male offspring from weight gain by increasing daytime energy expenditure (Wasinski et al., 2015). Specific epigenetic effects of maternal exercise protecting offspring from adverse effects of a maternal HF diet have been observed in that a maternal HF diet induced hypermethylation of the PGC-1 α promoter and reduced expression its target genes at birth with maintenance of the effect into adulthood (12 months of age). Maternal exercise ameliorated the effect of maternal HF diet on PGC-1 α hypermethylation and gene expression (Laker et al., 2014). In contrast, long-term paternal exercise may increase offspring risk to obesity and metabolic disease. Male mice that had engaged in 12 wk of wheel-running produced offspring with greater adiposity and insulin resistance, increased skeletal muscle expression of metabolic genes Slc2a, OGT, and Mgea5 (OGA), and decreased expression of PDK4, H19, and Ptpn1. These epigenetic effects may have occurred via exercise-induced alterations I gene methylation and mi-RNA content in the sperm of fathers (Murashov et al., 2016). The positive and negative phenotypic consequences of exercise on offspring adiposity and health may be a function of exercise producing epigenetic-based dose-

response hormesis (Chalk and Brown, 2014) with large volumes of at least paternal exercise producing offspring with a thrifty genotype (Murashov et al., 2016).

5. **Conclusions and Future Directions**

Epigenetic mechanisms that promote metabolic dysregulation and the onset of obesity and associated metabolic disease may be reversed by environmental factors such as dietary exercise, and pharmacological interventions that influence fetal development *in utero* (Figure1). Epigenetic changes influence the development of white, beige and brown adipocytes with varying rates of cellular energy metabolism, resulting in changes in body adiposity and risk for metabolic diseases. Future studies should address whether maternal aerobic exercise and increased consumption of bioactive nutrients in the diet can increase WAT mitochondrial biogenesis in offspring via epigenetic modifications. Additional studies are also needed to determine whether the beneficial effects of exercise or dietary nutrients on obesity and metabolic risk are transmitted to multiple generations, and the degree to which specific aspects of obesity-induced programming are temporary within and across generations. Lastly, studies are needed to determine whether the - environmental factors discussed above can modulate risk for metabolic disease through paternal lineage to affect offspring adipogenesis pathways.

FIGURE LEGENDS:

Figure 1: Maternal obesity, and exposure to environmental factors, can potentiate epigenetic modifications in the fetus. These include hypo (white lollipops) or hyper (filled lollipops) methylation of DNA; histone modifications (red circles=active marks, yellow circles=inactive marks) that can open or close chromatin near gene promoters; and changes in levels of non-

coding RNAs such as microRNAs. All these changes can act independently or in concert to affect changes in gene expression, turning networks of genes on or off in a tissue- and cell type- specific manner. These changes can increase offspring adiposity and risk for development of metabolic disease later in life. Conversely, these changes can be modulated by healthy diet, exercise and possibly pharmacological treatments, thereby decreasing adiposity and risk of metabolic disease in offspring.

FIGURE 1.

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