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Symposium 1: Nutrition and epigenetics DNA methylation of genes in adipose tissue

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Body fat distribution plays an important role in determining metabolic health. Whereas central obesity is closely associated with the development of CVD and type 2 diabetes, lower body fat appears to be protective and is paradoxically associated with improved metabolic and cardiovascular profiles. Physiological studies have demonstrated that fatty acid handling differs between white adipose tissue depots, with lower body white adipose tissue acting as a more efficient site for long-term lipid storage. The regulatory mechanisms governing these regional differences in function remain to be elucidated. Although the local microenvironment is likely to be a contributing factor, recent findings point towards the tissues being intrinsically distinct at the level of the adipocyte precursor cells (pre-adipocytes). The multi-potent pre-adipocytes are capable of generating cells of the mesenchymal lineage, including adipocytes. Regional differences in the adipogenic and replicative potential of these cells, as well as metabolic and biochemical activity, have been reported. Intriguingly, the genetic and metabolic characteristics of these cells can be retained through multiple generations when the cells are cultured *in vitro*. The rapidly emerging field of epigenetics may hold the key for explaining regional differences in white adipose tissue gene expression and function. Epigenetics describes the regulation of gene expression that occurs independently of changes in DNA sequence, for instance, DNA methylation or histone protein modification. In this review, we will discuss the contribution of DNA methylation to the determination of cells of adipogenic fate as well as the role DNA methylation may play during adipocyte terminal differentiation.

DNA Methylation: Adipogenesis: Body fat distribution

Body fat distribution and metabolic risk

The association between obesity and metabolic risk factors such as diabetes, dyslipidaemia and hypertension is well established. Body fat distribution, however, is highly variable and regional fat depots differ markedly in their contribution towards metabolic risk^(1,2). In human subjects, fat storage occurs in white adipose tissue (WAT) which is distributed throughout the body. The major WAT depots can be subdivided according to their regional location, into intra-abdominal (omental and visceral) and subcutaneous (abdominal, gluteal and femoral). Upper body, intra-abdominal WAT accumulation is typically associated with an increased risk of developing metabolic complications

whereas lower body, subcutaneous WAT is related to reduced risk. This paradoxical association has been the subject of much study^(3–5). Examining the relationship between body fat distribution and the risk of myocardial infarction, the INTERHEART study demonstrated a strong, independent relationship between increasing waist circumference (a measure of central adiposity) and myocardial infarction risk. In contrast, a trend for lower myocardial infarction risk was observed with increasing hip circumference⁽³⁾. Similar associations have also been reported for CHD⁽⁵⁾ and fasting plasma glucose levels⁽⁴⁾. The opposing effects of intra-abdominal and subcutaneous WAT depots are also evident in patients with partial lipodystrophy who display the selective loss of body fat.

Abbreviations: T-DMR, tissue-specific differentially methylated region; WAT, white adipose tissue.

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Typically born with normal fat distribution, patients with Dunnigan-type familial partial lipodystrophy display the selective loss of peripheral and truncal subcutaneous WAT around puberty while visceral and facial fat are preserved. Despite the loss of adipose tissue, such individuals display marked insulin resistance and have an increased predisposition to metabolic complications⁽⁶⁾. Conversely, the insulin-sensitising thiazolidinediones are associated with the redirection of fat accumulation from visceral to subcutaneous depots in patients with type 2 diabetes⁽⁷⁾. As reviewed by Manolopoulos *et al.*, the protective properties of lower body fat, particularly the gluteo-femoral depot, are likely to reflect the distinct metabolic profile of this tissue⁽²⁾. Local uptake and release of fatty acids differs considerably between WAT depots with upper body fat playing a more active role in the day-to-day uptake and release of diet-derived fatty acids^(8,9). In contrast, lower body fat appears to provide long-term entrapment of fatty acids^(10,11) and thus may protect insulin sensitive tissues, i.e. muscle, pancreas and liver, from ectopic fat accumulation.

Genetic determinants of regional body fat distribution

The existence of distinct body fat patterning in human subjects has long been recognised, but the precise contribution of genetic and environmental factors is less clear. Twin studies, which address the nature versus nurture debate, have repeatedly identified a strong genetic involvement in the development of overall obesity^(12–14). However, heritability estimates for body fat distribution have been highly variable, ranging from 6 to 89% when calculated for waist:hip ratios^(13,15). This has led some to conclude that environmental and behavioural factors exert a strong influence on body fat distribution⁽¹⁶⁾. Alternatively, this inconsistency can perhaps be explained by the inadequate sensitivity of many techniques that are used for measuring body fat distribution. For instance, when estimating abdominal obesity using indirect techniques such as waist:hip ratios, the heredity of body fat is reportedly as low as 6%⁽¹³⁾. In contrast, the same group reported a strong influence of genetic factors on regional fat distribution (85%) when truncal and lower body fat were determined by highly sensitive dual-energy X-ray absorptiometry imaging⁽¹⁴⁾. It would seem that when appropriate methodology is employed, body fat distribution can be viewed as highly heritable. By focusing on variation at the DNA level, common polymorphisms have been identified in several candidate genes known to be involved in lipid flux and metabolism in adipose tissue. These include the α 2-adrenoceptor and the glucocorticoid receptor, which were identified in the Quebec Family Study cohort⁽¹⁷⁾. Efforts to decipher the genetic basis of partial lipodystrophy have also identified genes that display defects resulting in the selective loss of WAT depots (lamin A/C, zinc metalloproteinase STE24, PPAR γ , protein kinase B, cell-death inducing Dffa-like effector C)^(6,18). At present, the spotlight has fallen on large, multi-centre, genome-wide association studies which aim to shed light on common genetic variation influencing body fat distribution.

Three novel loci (transcription factor AP 2B, methionine sulphoxide reductase A and lysophospholipase-like 1) have recently been implicated in body fat distribution by the meta-analysis of genome-wide association studies data from 38 580 individuals⁽¹⁹⁾. Disappointingly, the combined effects of these loci accounted for only 0.1% of the estimated genetic variation of the phenotype. Collectively, the genes identified to date explain only a small proportion of the variation in body fat distribution. There may be many more genes with similar effects which exert an additive effect on body fat distribution. Alternatively, rare variants with larger effect sizes may exist. However, it should be considered that a purely 'genetic-centred' view may not be able to provide all the answers and that alternative mechanisms (copy number variants, epigenetics) may explain some of the variation which has previously been ascribed as genetic.

Epigenetics and the developmental origins of white adipose tissue depots

Defined as 'the study of meiotically and mitotically heritable changes which are not caused by changes at the DNA level'⁽²⁰⁾, the epigenetic repertoire includes mechanisms that regulate transcription, such as DNA methylation and histone modification (methylation, acetylation, ubiquitination and sumoylation). During development, epigenetic signatures are acquired by differentiated cells and these play essential roles in maintaining cell identity through multiple cell generations. Adipose tissue, like bone and muscle, is believed to be derived from the mesoderm, but the mechanisms that orchestrate the transition from mesenchymal progenitor to adipocyte precursor remain largely unknown. Mesenchymal stem cells are fibroblast-like cells which develop from the mesoderm and are defined by their ability to give rise to adipose tissue, muscle, bone and connective tissue. It is thought that committed, undifferentiated precursor cells (e.g. pre-adipocytes) are initially generated and these cells then undergo terminal differentiation to generate mature, fully functional cells. A growing view is that different regions of the mesoderm may give rise to distinct populations of adipose precursor cells with distinct developmental roots. Essentially, each regional fat depot should be viewed as its own distinct mini-organ⁽²¹⁾. Unfortunately, few studies have investigated the embryological origin of WAT. Recently, the development of adipocytes from neural crest progenitors (having arisen from the ectoderm) has raised the possibility that WAT may not solely be of mesodermal origin⁽²²⁾.

At the cellular level, WAT is indeed extremely heterogeneous in nature. Precursor cells from different WAT depots (visceral, subcutaneous) are reported to differ in their capacity to replicate and differentiate⁽²³⁾, and adipocytes which are differentiated from these cells *in vitro* display discrete metabolic⁽²¹⁾ and genetic profiles^(24,25). An intriguing question that arises is how does an abdominal adipocyte distinguish itself from an omental or gluteal adipocyte and *vice versa*? All cells contain the same set of DNA, but through the expression and repression of specific

sets of genes they develop into highly specialised cells with unique characteristics⁽²⁶⁾. Complex developmental signalling systems (bone morphogenetic proteins, fibroblast growth factors and the Wnt signalling family) play important roles in adipose tissue development, as do other local factors such as innervation, nutrient supply and cell–cell interactions⁽²⁷⁾. However, a striking finding is that depot-specific characteristics are retained *in vitro* and can be handed down to subsequent daughter cells, in some cases for up to forty generations^(21,24). In other words, pre-adipocytes appear to retain an intrinsic memory of their regional location in the body. This finding strongly implicates epigenetic mechanisms in the establishment and maintenance of site-specific gene expression patterns in WAT.

The rapidly growing field of epigenetics presents an added dimension to explore in the study of gene regulation. Early experiments that utilised DNA demethylating agents drew attention to the potential importance of DNA methylation in the commitment of mesenchymal stem cells to the adipocyte lineage^(28,29). Treating rodent mesenchymal stem cell lines (C3H/10T1/2 or Swiss 3T3 cells) with the cytidine analogues, azacytidine or deoxyazacytidine, Taylor and Jones, reported the appearance of biochemically differentiated adipocytes⁽²⁸⁾. This differentiation was a stable event but was not specific, since myocytes and chondrocytes also developed. Studying the myocytes that arose, Jones and Taylor later reported a global reduction in DNA methylation in these cells compared to the mesenchymal progenitors from which they originated⁽²⁹⁾. These early studies suggested that in the multi-potent adipose precursor cells, DNA methylation maintained lineage-specific genes in a silenced state. In this review, we will focus on the role DNA methylation plays in establishing the cellular identity of WAT precursors, and how changes in DNA methylation may be involved in the terminal differentiation of adipocytes.

DNA methylation and tissue-specific methylation patterns

DNA methylation is classically associated with transcriptional repression that occurs as a result of changes in chromatin structure and protein–DNA interactions⁽³⁰⁾. In mammals, the methylation of DNA occurs at cytosine residues located within CpG dinucleotides⁽³¹⁾. The generation of 5-methylcytosine is brought about by a family of DNA methyltransferase enzymes, which catalyse the addition of methyl groups to cytosine⁽³⁰⁾. This reaction produces a stable modification that can regulate gene expression and cell differentiation and which is retained during cell division. CpG dinucleotides are unevenly distributed throughout the genome and the majority (about 80%) are maintained in a methylated state⁽³²⁾. However, concentrated CpG-rich regions, termed CpG islands, are typically found in an unmethylated state in normal cells. The exceptions to this are CpG islands on the inactive X-chromosome and those associated with imprinted genes, i.e. genes that are expressed differentially from the maternal or paternal allele. CpG islands are commonly observed

in the 5' region of genes where they span the promoter and untranslated regions. It has long been suggested that DNA methylation of promoter CpG islands may control tissue-specific gene expression; the prediction being that genes expressed in a tissue-specific manner would be hypermethylated in tissues where they are not expressed. Indeed, there is extensive evidence that some CpG islands display tissue-specific DNA methylation patterns^(33–35). However, since DNA methylation has not always been found to relate to gene expression this may be an over-simplistic view.

Using restriction landmark genomic scanning to probe methylation patterns in different tissues, Shiota *et al.* reported numerous tissue-specific differentially methylated regions (T-DMR) occurring throughout the genome⁽³³⁾. From their findings they estimated that some 4600 CpG islands (16% of total) may possess a T-DMR. This would imply that DNA methylation patterns at CpG islands are important epigenetic events that are involved in establishing and maintaining cell identity. A well characterised example is the human gene, *SERPINB5* (encoding mammary serine protease inhibitor, maspin), which displays an inverse correlation between tissue-specific expression and promoter methylation⁽³⁶⁾. In tissues where the *SERPINB5* promoter is hypermethylated (as it is in skin fibroblasts) the chromatin takes on a transcriptionally repressive conformation and the gene is not expressed. Treatment of fibroblasts with a demethylating agent such as azacytidine, however, enables the active expression of *SERPINB5* in these cells. Unfortunately, many studies have failed to demonstrate such definitive correlations between CpG island methylation status and the expression of tissue-specific genes^(34,35,37). The adipocyte lineage-specific genes leptin, *PPARγ2* and fatty acid binding protein 4 display promoter hypomethylation in mesenchymal stem cells isolated from not only adipose tissue but also muscle and bone marrow⁽³⁷⁾. Equally, muscle lineage-specific genes are hypomethylated in cells isolated from adipose tissue. It would seem that promoter hypermethylation may act as a definite barrier for gene expression whereas hypomethylation leaves a gene with the potential to be expressed but this is then subject to other regulatory mechanisms.

Although the predominant focus has been on CpG islands associated with the promoter regions of tissue-specific genes, T-DMR are widely distributed throughout the genome and can be found in introns, exons and non-genic regions^(38,39). Recently, it was estimated that 50% of T-DMR are not associated with 5' promoter CpG islands and this may point towards novel regulatory mechanisms of transcription⁽³⁵⁾. Indeed, DNA methylation upstream of the promoter in the gene body and transcriptional termination site has been positively correlated with gene expression⁽⁴⁰⁾. There is some evidence that non-promoter CpG islands may co-localise with the transcriptional start sites of non-coding RNA, which have important regulatory roles⁽³²⁾. T-DMR that affect the expression of non-coding RNA may therefore have indirect effects on the non-coding RNA targets.

Comparing the T-DMR profiles of different tissues can provide a measure of 'epigenetic distance'; in other words, it can give insight into how similar or dissimilar certain cell types are⁽³³⁾. Recently, an examination of

genome-wide DNA methylation patterns demonstrated that mesenchymal progenitor cells isolated from human adipose tissue have a similar DNA methylation pattern to the progenitor cells of bone marrow and skeletal muscle, supporting the notion of a common developmental origin. Notably, although the methylation patterns were very similar they were not identical, which suggests DNA methylation confers an inherent epigenetic identity⁽⁴¹⁾. It is tempting to speculate that precursor cells from different WAT depots may also display these unique epigenetic profiles. Unfortunately, evidence in support of this notion is limited. In rodents, brown and white pre-adipocytes, which for many years were thought to share a common developmental origin, display very similar but distinguishable methylation profiles⁽³⁸⁾. This is consistent with recent lineage-tracing experiments which have demonstrated that WAT and brown adipose tissue arise from independent precursors⁽⁴²⁾. Unfortunately, genome-wide studies to compare the epigenetic distance between precursors from different human WAT depots are currently lacking.

White adipose tissue depots and developmental gene expression

Gene expression profiling has consistently demonstrated that rodent and human WAT depots possess distinct regional gene expression signatures^(24,25,43,44). One of the most striking findings from these studies is the marked difference in expression of developmental and patterning genes. In human subjects, 25% of the transcripts that differ between pre-adipocytes isolated from subcutaneous abdominal and visceral WAT depots are related to embryonic development and the regulation of cell growth⁽²⁴⁾. Those genes displaying the greatest differences include short stature homeobox 2, engrailed-1 and members of the homeobox (*HOX*) and T-Box (*TBX*) families^(24,44). Although the importance of these developmental genes in WAT function remains unclear, several (*TBX15* and *HOXA5*) correlate with BMI and waist:hip ratios in human subjects⁽⁴⁴⁾. These observations suggest that developmental genes play important roles in WAT formation and distribution.

In other tissues, the contribution of *HOX* genes to cellular positioning and identity has been well characterised and multiple epigenetic mechanisms have been implicated in their transcriptional expression. *HOX* genes display a critical sequence of expression in the embryo and their continued expression is required in adult cells to maintain cellular positioning. DNA methylation and chromatin conformation are thought to be important regulators of this temporal-spatial expression pattern. In mammals, the *HOX* family consists of thirty-nine genes, which cluster in four chromosomal regions (*HOXA*, *HOXB*, *HOXC* and *HOXD*)⁽⁴⁵⁾. During development, the expression of *HOX* genes within each of these clusters occurs in a linear fashion, with the 3' genes generally being expressed earlier in development than the 5' genes. The position of a *HOX* gene within a cluster also correlates with expression along the anterior–posterior developmental axis; 3' genes

typically being expressed in anterior tissues and 5' genes in posterior tissues⁽⁴⁶⁾. Human fibroblasts taken from forty-three different regional locations possess site-specific *HOX* gene expression patterns, the so-called '*HOX* code', which appears to demarcate distinct positional boundaries⁽⁴⁷⁾. It would seem that this can also be applied to adipose tissue and there is growing evidence that a similar *HOX* code segregates WAT depots in different regional locations^(25,44,48).

The epigenetic regulation of *HOX* genes has received much attention and multiple mechanisms have been implicated in their transcriptional regulation. Developmental genes and, notably, members of the *HOX* family, are frequently associated with T-DMR^(35,49). Illingworth *et al.* compared CpG island methylation in blood, brain, muscle and spleen and found that CpG islands showing tissue-specific methylation were overrepresented at genetic loci associated with members of the *HOX* family⁽³⁵⁾. Although unmethylated in fetal tissue, *HOX* genes acquire tissue-specific methylation patterns in adult tissues⁽⁴⁹⁾. These tissue-specific DNA methylation patterns may serve as targets for histone modification to bring about further conformational changes in the chromatin to permanently switch *HOX* genes ON or OFF. The N-terminal tails of histone proteins undergo a number of biochemical modifications, in particular methylation. The Trithorax proteins catalyse the trimethylation of histone H3 at Lys⁴. Broad regions of the trimethylation of histone H3 at Lys⁴ exist in the *HOX* loci and display a direct correlation with the tissue-specific expression of *HOX* genes in fibroblasts from different regional locations⁽⁵⁰⁾. This suggests that the trimethylation of histone H3 at Lys⁴ is a mark of active chromatin domains, which is required for the maintenance of *HOX* gene expression⁽⁵⁰⁾. DNA methylation is thought to be intricately related to histone modification⁽⁵¹⁾ and a strong negative correlation exists between DNA methylation and trimethylation of histone H3 at Lys⁴⁽⁴⁰⁾. The *HOX* genes are an illustrative example of how DNA methylation and histone modification can interact to establish tissue-specific gene expression patterns which are critical in the establishment of distinct cell populations. Investigating the epigenetic status of these and other developmental genes in WAT depots will likely prove key to understand the development and positioning of WAT.

DNA methylation and terminal adipogenesis

As well as being implicated in the early determination of adipose precursor cells, changes in DNA methylation have also been observed during adipogenesis. This raises the possibility that DNA methylation is not merely a static, inherited state, but also a highly dynamic regulator of cellular differentiation which can be influenced by external stimuli. The transition of committed pre-adipocytes to functional adipocytes has been studied extensively *in vitro*. One particularly useful model for this has been the murine 3T3-L1 cell line. 3T3-L1 cells are fibroblast-like cells that are already committed to the adipocyte lineage and can be induced to differentiate following treatment with an adipogenic cocktail which typically

contains insulin, dexamethasone and 3-isobutyl-1-methyl-xanthine⁽⁵²⁾. In contrast to mesenchymal stem cells⁽²⁸⁾, the treatment of 3T3-L1 cells with azacytidine inhibits adipocyte differentiation in a stage-dependent manner⁽⁵³⁾. This supports the view that the establishment of distinct DNA methylation patterns is required for terminal adipocyte differentiation. By examining a subset of known T-DMR in 3T3-L1 cells, Sakamoto *et al.* identified a small proportion of these which displayed changes in DNA methylation during differentiation; notably one of those to change was a T-DMR associated with the *HOXC6* gene. Additionally, thirty-two novel T-DMR were identified by comparing 3T3-L1 cells at different stages of differentiation. The changes in methylation which occurred during differentiation were both unidirectional and transient and involved hyper- and hypomethylation, suggesting a complex and dynamic underlying component to adipogenesis⁽⁵³⁾.

Terminal adipocyte differentiation is tightly regulated by a cascade of transcription factors (e.g. CCAAT/enhancer binding protein α , *PPAR* γ) which are expressed in a coordinated fashion and lead to the expression of adipocyte-specific genes⁽⁵²⁾. Despite evidence that changes in DNA methylation influences adipogenesis, very few studies have investigated the connection between DNA methylation and transcriptional regulation of adipogenesis. Distinct CpG sequences within the promoter regions of the late adipogenic genes leptin and GLUT4 display demethylation during adipogenesis which corresponds with the expression of these genes in mature adipocytes^(54,55). In the case of leptin, the binding of methyl-CpG-binding proteins, resulting in chromatin remodelling, appears to contribute to the transcriptional silencing of this gene when it is methylated. More recently, methylation of the *Wnt10b* gene promoter has been shown to prevent binding of the adipogenic transcription factor cyclic AMP responsive element-binding protein, thereby decreasing *Wnt10b* expression⁽⁵⁶⁾. *Wnt10b* is expressed in pre-adipocytes and is thought to inhibit the initiation of adipogenesis. Intriguingly, methylation and the subsequent silencing of *Wnt10b* appear to occur via a cyclic AMP-dependent mechanism that allows adipogenesis to proceed. How cyclic AMP triggers promoter methylation remains uncertain and it is not known whether other adipogenic genes may also be regulated in this manner.

Concluding remarks

Body fat distribution plays an important role in determining metabolic health. This is intricately linked to the differential capacity of regional WAT depots to store and release fatty acids. Evidence emerging from *in vitro* studies strongly suggests that distinct populations of adipose precursor cells contribute to the functional heterogeneity of WAT. Epigenetic modification, and in particular DNA methylation, plays an essential role in cellular determination and differentiation. Numerous tissue-specific differentially methylated regions have been identified throughout the genome and can be used to distinguish mesenchymal stem cells derived from adipose tissue and other

mesodermal tissues. However, since these regions often fail to correlate with transcriptional activity and differentiation capacity it would seem that novel regulatory mechanisms (micro RNA and non-coding RNA) remain to be fully explored. Whether regional WAT depots in human subjects display unique epigenetic profiles is another important question still to be addressed. The observation that WAT precursor cells retain regional characteristics certainly agrees with the notion that these cells possess an inherent epigenetic memory, and raises the intriguing possibility of novel applications, such as adipose tissue transplantation⁽⁵⁷⁾, to promote the metabolically protective effects of certain WAT depots.

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