Epigenetic regulation of diabetogenic adipose morphology

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ABSTRACT

Objective: Hypertrophic white adipose tissue (WAT) morphology is associated with insulin resistance and type 2 diabetes. The mechanisms governing hyperplastic versus hypertrophic WAT expansion are poorly understood. We assessed if epigenetic modifications in adipocytes are associated with hypertrophic adipose morphology. A subset of genes with differentially methylated CpG-sites (DMS) in the promoters was taken forward for functional evaluation.

Methods: The study included 126 women who underwent abdominal subcutaneous biopsy to determine adipose morphology. Global transcriptome profiling was performed on WAT from 113 of the women, and CpG methlyome profiling on isolated adipocytes from 78 women. Small interfering RNAs (siRNA) knockdown in human mesenchymal stem cells (hMSCs) was used to assess influence of specific genes on lipid storage.

Results: A higher proportion of CpG-sites were methylated in hypertrophic compared to hyperplastic WAT. Methylation at 35,138 CpG-sites was found to correlate to adipose morphology. 2,102 of these CpG-sites were also differentially methylated in T2D; 98% showed directionally consistent change in methylation in WAT hypertrophy and T2D. We identified 2,508 DMS in 638 adipose morphology-associated genes where methylation correlated with gene expression. These genes were over-represented in gene sets relevant to WAT hypertrophy, such as insulin resistance, lipolysis, extracellular matrix organization, and innate immunity. siRNA knockdown of ADH1B, AZGP1, C14orf180, GYG2, HADH, PRKAR2B, PFKFB3, and AQP7 influenced lipid storage and metabolism.

Conclusion: CpG methylation could be influential in determining adipose morphology and thereby constitute a novel antidiabetic target. We identified C14orf180 as a novel regulator of adipocyte lipid storage and possibly differentiation.

Keywords Adipocyte; Epigenetics; Adipogenesis; Insulin resistance

1. INTRODUCTION

White adipose tissue (WAT) dysfunction is a hallmark of obesity and associated metabolic complications such as type 2 diabetes (T2D) [1]. WAT expands by increasing adipocyte number (hyperplasia) and size (hypertrophy). Their relative importance differs between individuals resulting in alternate adipose morphologies. Hypertrophic WAT is the pernicious morphology, which is associated with dyslipidemia, insulin resistance, and T2D [2–5]. The increased incidence of metabolic syndrome observed with hypertrophic WAT may be explained by higher spontaneous lipolysis and release of pro-inflammatory adipokines [6,7].

The mechanisms governing hyperplastic versus hypertrophic WAT expansion are poorly understood. Family history of diabetes is associated with enlarged adipocytes supporting a genetic impact on adipose morphology [8]. Furthermore, WAT hypertrophy has been linked to altered extracellular matrix function and impaired adipogenesis [9,10]. The observation that generation of new adipocytes is constant during adulthood [11] gives support to the notion that long acting, e.g. epigenetic, mechanisms could participate in regulation of adipose morphology. One epigenetic mechanism by which cells are able to modulate their gene expression is DNA methylation at CpG dinucleotides [12]. In particular, tissue specific methylation in gene promoters inhibits gene expression [13]. Previous studies have compared CpG-methylation in WAT specimen between insulin resistant and sensitive, or T2D and non-diabetic subjects [14,15]. However, a minority of cells in WAT constitutes adipocytes, the cell type defining adipose morphology [16]. To directly address the question if differentially methylated CpG-sites (DMS) in adipocytes are associated with adipose morphology and adipocyte function, we here report CpG-methylyme profiling on abdominal subcutaneous adipocytes from a large cohort of women. CpG-methylation was related to adipose morphology and gene expression analyzed on the same biopsies. A subset of genes with DMS in the promoters were taken forward for functional evaluation by small interfering RNAs (siRNA) knockdown in adipose derived human...
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mesenchymal stem cells (hMSCs) to assess influence on lipid metabolism and storage.

2. METHODS

2.1. Patient examination

The study included 126 women recruited by local advertisement from the general adult population in the Stockholm (Sweden) area (Table 1); 113 women underwent gene expression and 78 CpG methylation profiling. The women displayed a large inter-individual variation in body mass index (BMI), and all were healthy. Those in the gene expression group have been described previously [17]. Participants were investigated at 8 AM after an overnight fast. Anthropometric and blood pressure measurements were performed followed by venous blood sampling. Fasting plasma glucose and lipids were analyzed at the hospital’s routine chemistry laboratory. Serum insulin was measured by ELISA (Merckodia, Uppsala, Sweden). Homeostasis model assessment of insulin resistance (HOMA-IR) was computed according to Matthews et al. [18]. Following the clinical examination an abdominal subcutaneous WAT biopsy was obtained by needle aspiration as described [19]. The study was approved by the regional ethics board in Stockholm, and written informed consent was obtained from each subject. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. WAT experiments

WAT specimens were subjected to collagenase treatment to obtain isolated adipocytes as described [20]. One portion of cells was cultured in growth media for quantitation of adipocyte number as described[19]. The study was approved by the regional ethics board in Stockholm, and written informed consent was obtained from each subject. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.3. Gene expression profiling

Global transcriptome profiling on WAT specimen was performed using GeneChip® Human Transcriptome Array 2.0 (Affymetrix) according to the manufacturer’s instructions. RNA was isolated as described in section 2.8. Pre-processing and normalization of arrays were carried out with Affymetrix Expression Console 4.0 (Affymetrix) using Gene Level SST-RMA.

2.4. DNA preparation

Genomic DNA was prepared from isolated adipocytes using the QiAamp DNA Mini kit (cat no. 51304, Qiagen, Hilden, Germany). DNA purity and quality was confirmed by A260/280 ratio >1.8 on a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). DNA concentration was measured by Qubit (Life technologies, Stockholm, Sweden).

2.5. CpG methylation profiling

Global CpG-methylation profiling was performed on isolated adipocytes using the Infinium Human Methylation EPIC BeadChip (Illumina, San Diego, CA) as described in Appendix A. During pre-processing we removed probes overlapping SNPs, with unreliable measurement criteria (P > 0.05), and 2,638 context-specific probes leaving 820,027 probes for further analysis. Methylation levels (beta values; β) were estimated as the ratio of signal intensity of the methylated alleles to the sum of methylated and unmethylated intensity signals. The β-values vary from 0 (no methylation) to 1 (100% methylation).

2.6. Microarray analyses

Quilcrome Omics Explorer 3.2 (www.quilcrome.com) was used for micro-array analyses of clinical samples. Before phenotypic analysis, the 22,167 transcripts on the Gene 1.0 and 1.1 ST arrays were collapsed on to 20,506 unique transcripts by selecting probe-sets with highest average expression for each gene. Relationship between gene expression and adipose morphology was assessed by linear regression adjusting for array batch. We used Webgestalt (www.webgestalt.org, accessed 1/12-2018) and the KEGG and REACTOME databases to assess overrepresented gene sets among differentially expressed genes, as compared to all mRNA in the human genomes. Phenotypic analysis of EPIC arrays was limited to the 571,997 CpG-sites mapping to a unique site and linked to a transcript. Relationship between CpG-methylation beta-values and WAT morphology was assessed by linear

| Table 1 – Clinical characteristics of studied women. |
|------------------------------------------------------|
| **CpG methylation in adipocytes**                     |
| Hypertrophy (n = 42)                                  |
| Hyperplasia (n = 36)                                  |
| *P*                                                  |
| Age (y’s)                                            |
| 45 ± 13                                              |
| 39 ± 11                                              |
| 0.045                                                |
| BMI (kg/m²)                                          |
| 33 ± 9                                               |
| 29 ± 9                                               |
| 0.021                                                |
| Systolic blood pressure (mm Hg)                      |
| 129 ± 15                                             |
| 124 ± 19                                            |
| 0.13                                                |
| Diastolic blood pressure (mm Hg)                     |
| 78 ± 7                                               |
| 75 ± 9                                               |
| 0.076                                                |
| IF-Glucose (mmol/L)                                  |
| 5.17 ± 0.47                                          |
| 4.72 ± 0.40                                          |
| 8 × 10⁻⁵                                            |
| IS-Insulin (ml/L)                                    |
| 11.98 ± 8.87                                         |
| 5.77 ± 3.99                                         |
| 3 × 10⁻⁵                                            |
| HOMA-IR                                              |
| 2.82 ± 2.25                                         |
| 1.21 ± 0.85                                         |
| 8.6 × 10⁻⁵                                          |
| Spontaneous lipolysis (µmol glycerol/2 h/g lipid)     |
| 7.4 ± 6.7                                            |
| 3.3 ± 3.0                                            |
| 0.0004                                              |
| Total cholesterol, mmol/l                            |
| 5.16 ± 0.99                                          |
| 4.52 ± 0.84                                         |
| 0.0038                                              |
| IF-HDL cholesterol, mmol/l                           |
| 1.36 ± 0.39                                          |
| 1.61 ± 0.40                                         |
| 0.004                                               |
| IF-Triglycerides (mmol/L)                            |
| 1.51 ± 0.85                                          |
| 0.92 ± 0.49                                         |
| 0.0004                                              |
| Adipose morphology (delta)                           |
| 145 ± 114                                            |
| −122 ± 98                                            |
| 4 × 10⁻¹⁴                                           |
| Gene expression in WAT                               |
| Hypertrophy (n = 60)                                  |
| Hyperplasia (n = 53)                                  |
| *P*                                                  |
| Age (y’s)                                            |
| 44 ± 12                                              |
| 41 ± 10                                              |
| 0.13                                                |
| BMI (kg/m²)                                          |
| 34 ± 9                                               |
| 33 ± 10                                              |
| 0.43                                                |
| Systolic blood pressure (mm Hg)                      |
| 128 ± 17                                             |
| 127 ± 17                                            |
| 0.91                                                |
| Diastolic blood pressure (mm Hg)                     |
| 78 ± 8                                               |
| 77 ± 9                                               |
| 0.14                                                |
| IF-Glucose (mmol/L)                                  |
| 5.32 ± 0.72                                          |
| 4.99 ± 0.53                                         |
| 0.011                                               |
| IS-Insulin (ml/L)                                    |
| 12.79 ± 9.16                                        |
| 8.29 ± 6.28                                         |
| 0.0028                                              |
| HOMA-IR                                              |
| 3.09 ± 2.36                                         |
| 1.89 ± 1.59                                         |
| 0.0011                                              |
| Total cholesterol, mmol/l                            |
| 5.18 ± 0.85                                          |
| 4.51 ± 0.86                                         |
| 2.7 × 10⁻⁵                                          |
| IF-HDL cholesterol, mmol/l                           |
| 1.36 ± 0.40                                          |
| 1.49 ± 0.39                                         |
| 0.048                                               |
| IF-Triglycerides (mmol/L)                            |
| 1.57 ± 0.86                                          |
| 1.05 ± 0.61                                         |
| 0.0002                                              |
| Adipose morphology (delta)                           |
| 143 ± 106                                            |
| −121 ± 93                                           |
| 6 × 10⁻²⁰                                           |

* Difference between average fat cell volume and expected volume for specific total body fat, see methods for detail.

* Wilcoxon test was used to compare clinical and biochemical variables between groups. Values are mean ± SD.
2.11. De novo lipogenesis
from sample results. Fresh media not exposed to cells was used as blank and subtracted (buffer). The decay in luminescence over 1 min was measured and Table 2. Gene expression was normalized to donor (16 years old, BMI 24 kg/m²). hMSCs were cultured in 24 hMSCs were isolated from abdominal subcutaneous WAT from a male samples using the MSigDB Hallmark gene set collection\[25,26\].

2.7. Cell culture experiments
hMSCs were isolated from abdominal subcutaneous WAT from a male donor (16 years old, BMI 24 kg/m²). hMSCs were cultured in 24 (1 × 10⁵ cells/well) or 48-well (5 × 10⁴ cells/well) plates and differentiated into adipocytes using described protocols [27]. For gene knockdown at day -1, hMSCs were transfected with small interfering RNAs (siRNA) targeting a candidate gene (Dharmacon™ OnTarget plus SMART pool or Dharmacon™ siGENOME SMART pool, WVR International, Sweden) or negative control siRNA (siNC) (Dharmacon™ siGENOME Control Pool) using DharmaFECT™ 3 transfection reagent (WVR International) as per manufacturer’s instructions. For transfection at day 8 a Neon™ transfection system (MPK5000, Invitrogen, Göteborg, Sweden) was used as per manufacturer’s instructions. Details of siRNAs used are given in Appendix B, Supplemental Table 1.

2.8. qRT-PCR
On day 9 of hMSCs differentiation, cells were harvested and RNA isolated using the RNeasy Mini Kit (217004, Qiagen). RNA concentration and purity was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (1708891, Bio-Rad, Sundbyberg, Sweden) followed by SYBR green qRT-PCR analysis on an iCycler IQ (Bio-Rad). Primer sequences are given in Appendix B, Supplemental Table 2. Gene expression was normalized to β2M using the delta delta Ct method.

2.9. Lipid droplet quantification
On day 9 of hMSC differentiation cells were fixed in 4% paraformaldehyde for 10 min before washing in PBS. Lipid was stained using BODIPY™ (0.2 μg/ml) (Fisher Scientific, Göteborg, Sweden) and nucleus with Hoechst 33258 (10 mg/mL) (Fisher Scientific). Images were taken after total lipid per cell and total lipid droplets per cell were quantified on a CellInsight CX5 High Content Screening Platform (Fisher Scientific).

2.10. Rate of lipolysis
Basal lipolysis was determined by measuring the concentration of glycerol in the media after three days incubation as previously described [28]. Briefly, at day 13 of differentiation, 50 μL of media was collected and added to 450 μL of resuspended ATP reagent (10 μL purified glycerokinase (Sigma–Aldrich, Stockholm, Sweden) and 10 μL ATP Reagent SL (Biotherma, Handen, Sweden) in 0.05 M Tris buffer). The decay in luminescence over 1 min was measured and compared with a glycerol standard curve to determine concentration. Fresh media not exposed to cells was used as blank and subtracted from sample results.

2.11. De novo lipogenesis
Incorporation of [1-¹⁴C]-acetic acid (Perkin Elmer, Stockholm, Sweden) into hMSCs was analyzed as previously described [29]. On day 13 of differentiation cells were insulin starved for 18 h in DMEM media containing additives as described [27] and 1 μM glucose. Following starvation, cells were incubated in DMEM media containing 2 mM glucose, 10 mM HEPES, 0.2% BSA, 2 mM acetate and 1 μCi [1-¹⁴C]-acetic acid with or without 100 nM insulin. Cells were lysed in 0.1% SDS and radioactivity in lysate determined on a scintillation counter.

2.12. Statistical analyses
Wilcoxon test was used to compare clinical and biochemical variables between groups. Student’s t test (2 sided) was used to compare the global average proportion of methylated CpG-sites between the two clinical groups, and the average difference in methylation of DMS between adipose morphology and T2D. Relationship between CpG-methylation and gene expression was assessed by Pearson correlation. Chi-square test was used to compare distribution of DMS between groups. A one-way ANOVA test with a Dunnett’s multiple comparison test was used to test for significance between treatment and control samples in cell culture experiments.

3. RESULTS
3.1. Clinical characteristics of study participants
Seventy-eight women underwent CpG-methylation profiling on isolated adipocytes, and 113 women underwent transcriptome profiling on WAT; 65 subjects overlapped between these analyses (Table 1). Subjects were grouped based on adipose morphology. WAT hypertrophy was associated with a pnenic metabolic profile with higher plasma fasting glucose, fasting insulin, and HOMA-IR, as well as total cholesterol and triglycerides. Adipocyte spontaneous lipolysis was also higher in the subgroup with WAT hypertrophy. In the CpG-methylation cohort age and BMI were slightly higher in the subjects with WAT hypertrophy as compared to those with WAT hyperplasia, whereas no difference in these parameters was observed between WAT hypertrophy and hyperplasia in the transcriptome cohort.

3.2. Transcriptome profile in relation to WAT morphology
Global transcriptome analysis on WAT identified 1,131 genes whose expression significantly correlated with adipose morphology (FDR ≤ 0.01; Appendix B, Supplemental Table 3). The 1,131 genes were enriched for gene sets relevant to WAT hypertrophy including Regulation of lipolysis rate, Insulin resistance, Adipocytokine signaling, Extracellular matrix organization, and Neutrophil degranulation (FDR < 0.15; Appendix B, Supplemental Table 4). The genes assigned to each pathway and correlated to adipose morphology are presented in Appendix B, Supplemental Table 5. Examples of genes in metabolic pathways inversely correlated with adipose morphology include IRS2, a central player in insulin signaling (FDR < 0.01, R = -0.35). Expression of genes in pro-inflammatory responses such as IL1RN (FDR < 0.01, R = 0.39), CCL22 (FDR < 0.01, R = -0.35), and CCR1 (FDR < 0.01, R = 0.34) correlated positively with adipose morphology.

3.3. CpG-methylation landscape in relation to WAT morphology
We assessed methylation at 820,026 distinct CpG-sites in relation to adipose morphology. There was a borderline significant overall difference in CpG-methylation between women with hypertrophic versus hyperplastic WAT (Table 2, P = 0.058). However, in and around the transcription start sites (TSS) of genes [TSS 1500, TSS 200, 5’ untranslated region (UTR) and 1st Exon] there was a significantly higher proportion of methylated CpG-sites in hypertrophic compared to hyperplastic WAT. Significant differences were not observed for other gene regions examined. In relation to CpG islands, significant alterations in the methylation state between the adipose morphologies occurred within CpG islands and surrounding shores. Methylation at 35,138 CpG-sites were found to significantly correlate to adipose morphology at a FDR ≤ 0.01 (Appendix B, Supplemental
Global CpG methylome signature in relation to adipose morphology. 2,061 (98%) showed directionally consistent change in methylation in 2,102 CpG-sites were associated with WAT morphology of which associated with T2D [15]. Among 15,627 DMS in WAT associated with T2D, 3.4. DMS common to WAT hypertrophy and T2D open sea regions, and under-representation in CpG islands. The genome distribution of the DMS asso-
ciated with adipose morphology signi

3.4. DMS common to WAT hypertrophy and T2D We next compared the 35,138 DMS associated with adipose morphology in the present study with previously reported DMS associated with T2D [15]. Among 15,627 DMS in WAT associated with T2D, 2,102 CpG-sites were associated with WAT morphology of which 2,061 (98%) showed directionally consistent change in methylation in WAT hypertrophy and T2D (Appendix B, Supplemental Table 6). Differential methylation at DMS was overall more pronounced in isolated adipocytes in relation to adipose morphology as compared to in WAT in relation to the clinical cohort, and [3] expression enriched in adipocytes. For the latter assessment, we used the FANTOM5 project database (http:// fantom.gsc.riken.jp/) which interrogates the expression level of genes in various primary cell types including our adipose derived hMSCs across differentiation. The 11 selected genes displayed the highest expression and largest fold increase during differentiation into adipocytes, as well as high expression in mature adipocytes compared with other tissue and cells.

To examine if the 11 adipose morphology associated genes influenced adipocyte function, their expression was knocked down in hMSCs at the early (day -1) and late (day 8) stage of differentiation into adipocytes. Each siRNA was able to markedly and signi

3.5. Identification of differentially methylated genes associated with adipose morphology To identify epigenetically regulated genes of potential importance for adipose morphology we overlapped the 35,138 DMS with the 1,131 differentially expressed genes and hereby identified 2,508 DMS in 638 genes where methylation correlated with expression of the corresponding gene with an $\rho \geq 0.6$ (Appendix B, Supplemental Table 7). The 638 genes were over-represented in gene sets relevant to WAT hypertrophy, such as Insulin resistance (e.g. AKT2, PIK3CG, SLC2A4, and IRS2), Regulation of lipolysis in adipocytes (e.g. ADRB1, ADRB2, ABHD5), Extracellular matrix organization (e.g. collagen, cathepsins, and integrins), and Innate Immune System (Appendix B; Supplemental Table 8).
knockdown of AQP7 stimulated lipid storage but caused blunted the response to insulin (Figure 1G). Gene knockdown had modest effect on basal lipogenesis (Figure 1G). siRNA knockdown of C14orf180 had no effect on any investigated phenotype.

3.7. C14orf180 is critical for adipogenic gene expression
Among the candidate genes for adipose morphology from the siRNA experiment, only C14orf180 significantly blunted all examined phenotypes. As little is known about the function of C14orf180, we carried out global transcriptome analysis on hMSCs transfected with siC14orf180 or siNC at day -1 of differentiation. 1,116 genes were differentially expressed between these groups at a FDR $<0.01$ (Appendix B, Supplemental Table 9). C14orf180 knockdown down-regulated expression of genes in pathways such as Adipogenesis and Oxidative phosphorylation and upregulated genes in the interferon-α pathway (FDR $<0.25$) (Figure 1H, Appendix C, Supplemental Figure 1).

Specific genes downregulated in siC14orf180 treated cells include the master regulators of adipogenesis PPARγ and CEBPa, as well genes essential for lipid metabolism (LIPE, LPL, FABP4, PLIN2, and DGAT1) (Appendix B, Supplemental Table 9).

4. DISCUSSION
In this study, we present results linking adipocyte epigenetic profile to adipose morphology. In a genome-wide analysis, we demonstrate a modest but significant increase in proportion of methylated CpG-sites in the 5’ regions of genes, as well as in CpG islands and surrounding shore regions in adipocytes from women with hypertrophic WAT. Among DMS overlapping between WAT hypertrophy and T2D, 98% display concordant change in CpG-methylation between these clinical conditions. 638 genes display correlation between promoter CpG-methylation, expression, and adipose morphology, including genes

| Table 3 – Summary of selected gene displaying correlation between promoter CpG methylation, expression, and adipose morphology. a |
| --- |
| Gene | CpG b | Adipose morphology | Gene expression | effect of siRNA on hMSCs c | Concordant DMS in T2D |
| --- | --- | --- | --- | --- | --- |
| APLP1 | cg08859770 0.0012 | 0.38 | 0.034 | 0.25 | down down down yes |
| AQP7 | cg16447312 0.0012 | 0.48 | 0.0008 | 0.41 | down down down blunted |
| AZGP1 | cg07059704 0.0009 | 0.47 | 0.0016 | 0.2 | down down down blunted |
| BCL2L11 | cg15665105 0.0014 | 0.47 | 0.0016 | 0.2 | down down down blunted |
| C14orf180 | cg00497078 0.0009 | 0.49 | 0.0016 | 0.2 | down down down blunted |
| CAMK1 | cg02868468 0.0012 | 0.48 | 0.0001 | 0.48 | down down yes |
| CAMK1 | cg10836985 0.0008 | 0.48 | 0.0001 | 0.48 | down down yes |
| CAMK1 | cg22052044 0.0008 | 0.48 | 0.0001 | 0.48 | down down yes |
| CAMK1 | cg01573501 0.0008 | 0.48 | 0.0001 | 0.48 | down down yes |
| CAMK1 | cg03889890 0.0008 | 0.48 | 0.0001 | 0.48 | down down yes |

a Adipose morphology was assessed as difference between average fat cell volume and expected volume for specific total body fat. see methods for detail. For selection of genes see results section.

b All CpG sites listed below are located within 1500 basepairs from transcription start site.

c Relationship between gene expression and adipose morphology was assessed by linear regression adjusting for beta-values, and adipose morphology was assessed by linear regression adjusting for array batch.

d Relationship between CpG-methylation, as assessed by beta-values, and adipose morphology, was assessed by linear regression adjusting for age.

e Pearson r 2.

f Genes in the leftmost column were knocked down by siRNA and effect on lipid accumulation and gene expression assessed as described in method section.

g Specific genes downregulated in siC14orf180 treated cells include the master regulators of adipogenesis PPARγ and CEBPa, as well genes essential for lipid metabolism (LIPE, LPL, FABP4, PLIN2, and DGAT1) (Appendix B, Supplemental Table 9).
versus target siRNA (C20). A decrease in proportion of methylated Cpg-sites in adipocytes [32,33].

phenotype with small adipocytes, which is associated with a global to, total body fat [23]. Bariatric surgery induces a hypercellular WAT adipose morphology applied herein is adjusted for, and thus not related causality between adipose morphology and diabetes. The measure of strong overlap with those observed in T2D further strengthening the study, adipocyte DMS associated with adipose hypertrophy display predisposing hypertrophy, are poorly understood. In the present factors regulating adipose morphology, in particular diabetes-related to insulin resistance, regulation of lipolysis, and extracellular matrix organization. By siRNA knockdown we identify, among differentially methylated genes, novel regulators of adipocyte lipid storage including C14orf180.

The factors regulating adipose morphology, in particular diabetes-predisposing hypertrophy, are poorly understood. In the present study, adipocyte DMS associated with adipose hypertrophy display strong overlap with those observed in T2D further strengthening the causality between adipose morphology and diabetes. The measure of adipose morphology applied herein is adjusted for, and thus not related to, total body fat [23]. Bariatric surgery induces a hypercellular WAT phenotype with small adipocytes, which is associated with a global decrease in proportion of methylated Cpg-sites in adipocytes [32,33]. This is concordant with the finding in the present study where hypertrophic WAT is associated with an increase in global Cpg-methylation in 5’ regions of genes, and gives support to the notion that adipocyte DNA methylation is causally linked to cell size. Life style interventions with physical exercise and altered dietary fatty acid composition have been shown to influence WAT Cpg-methylation [34,35], but the impact on the adipocyte epigenome or adipose morphology has, to our knowledge, not been investigated. Most previous epigenome profiling on WAT have studied tissue specimen. Adipocytes constitute a minority of cells in WAT, which also comprise fibroblasts, endothelial, immune, and precursor cells [36]. In the present study, we examined only the mature adipocytes and, in this subpopulation of cells in WAT, identified numerous genes displaying

Figure 1: Impact of candidate gene knockdown in adipose derived human mesenchymal stem cells (hMSCs) on lipids per cell, gene expression and lipid turnover. siRNAs targeting the indicated genes or siNegative Control (siNC) were transfected on Day -1 (A–E and H) or on Day 8 (F–G) of differentiation alongside a Mock transfection. For Day -1 knockdown, cells were lysed or fixed and mRNA or lipid was quantified on day 9 of differentiation. For day 8 knockdown, glycerol in the media or lipid synthesized in the cells was measured at day 13. A. mRNA quantification of the siRNA target gene compared with mock in transfected cells. B. Total lipid per cell or C. Total lipid droplets per cell were quantified after lipid staining and normalized per nucleus. n = 3 per group. D. ADIPOQ or E. FABP4 mRNA quantification after target gene knockdown as indicated on the x-axis. n = 3–7 per group. F. Basal lipolysis measured as glycerol release into the media. De novo lipogenesis over 3 h with or without the addition of insulin. Lipogenesis was measured via radiolabeled acetate incorporation into the cell. H. Global transcriptome profiles on siC14orf180 or siNC transfected samples were subjected to Gene Set Enrichment Analysis (GSEA). The upper panel shows gene sets downregulated and the lower panel gene sets upregulated by siC14orf180 knockdown compared to siNC. Statistical analysis carried out for B-F was target siRNA versus siNC. Statistical analysis for G carried out was (−) insulin versus (−) insulin significance denoted by * and siNC (−) insulin versus target siRNA (−) insulin significance denoted by #. One-way ANOVA with Dunnetts post hoc test was carried out for all experiments shown. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, #P ≤ 0.05, ###P ≤ 0.001.
The correlation between promoter CpG-methylation, expression, and adipocyte morphology. DMS associated with WAT hypertrophy were over-represented outside CpG islands, regions where methylation is thought to be more dynamic and tissue specific, as compared to CpG islands [12,13]. CpG-sites in the vicinity of the transcription start site are of particular importance for cell function since their methylation prevent initiation of transcription [12]. Inverse correlation between adipocyte promoter CpG-methylation and WAT gene expression in vivo can be validated in vitro, i.e. methylation at such CpG-sites inhibits gene expression [37]. This supports the notion that at least some of the genes reported here and related to e.g. insulin resistance, regulation of lipolysis, and extracellular matrix organization are linked to adipocyte function and WAT hypertrophy. A subset of genes with DMS was taken forward for functional evaluation by siRNA knockdown in hMSCs and were shown to influence lipid storage and turnover thus representing new potential regulators of adipose morphology (Table 3):

- **C14orf180** has been reported to encode a plasma-membrane protein with unknown function displaying enriched expression in WAT [38]. We report that knockdown of **C14orf180** inhibits lipid storage in hMSCs. **C14orf180** deficiency downregulated expression of key genes in adipogenesis including **PPARG** and **CEBP**A, as well as numerous genes involved in lipid metabolism. The interferon-α pathway, which was upregulated upon **C14orf180** knockdown, has previously been shown to inhibit adipogenesis [39]. Thus, we identified **C14orf180** as a novel regulator of adipocyte lipid storage and possibly differentiation. **C14orf180** displays numerous DMS in the promoter region, one of which overlaps with T2D (Table 3). Together these findings support the notion that epigenetic regulation of **C14orf180** contributes to diabetogenic hypertrophic WAT.

- **AZGP1** encodes the adipokine ZAG1, which has been reported to reduce body weight and expression of lipogenic enzymes in WAT of rodents [40]. The latter is in contrast to our results in which knockdown of **AZGP1** inhibited lipid storage and blunted insulin stimulated lipolipogenesis. The discrepant results could be due either to reduced lipid storage in the present study being secondary to reduced adipogenesis, as supported by reduced ADIPOQ expression, or species specific effects of **AZGP1**.

- **PFKFB3** inhibition impairs basal and insulin stimulated lipolipogenesis, and gene knockdown may inhibit adipocyte lipid storage as observed in the present study [41].

- **PKAR2B** is required for adipogenesis and low levels are associated with increased lipolysis [42,43]. Consistent with these findings, in the present study, **PKAR2B** knockdown inhibited lipid accumulation and increased basal lipolysis. DMS in the **PKAR2B** promoter region overlap between WAT hypertrophy and T2D thus supporting the notion that epigenetic regulation of **PKAR2B** predisposes to hypertrophic WAT and T2D.

- Genetic variants in **ADH1B** affect body fat [44] consistent with **ADH1B** knockdown inhibiting lipid storage.

- **HADH** is involved in beta oxidation and **GYG2** controls glycerogen storage. Our findings that **HADH** and **GYG2** knockdown inhibits lipid storage adds novel metabolic function to these genes. **GYG2** knockdown also increased basal lipogenesis perhaps resulting from decreased free glycerogen levels which has been shown to increase glucose uptake in adipocytes [45].

- **AQP7** was the only gene whose knockdown increased lipid storage in hMSCs. This effect is consistent with **AQP7** encoding an adipocyte membrane protein responsible for glycerol transport, a substrate and product of lipid metabolism [46].

WAT hypertrophy has been shown to be caused by impaired recruitment of new adipocytes and epigenetic factors control adipogenesis [10,47]. We do not have samples to directly study the precursor cell epigenome in clinical cohorts. Therefore, it is unclear if DMS adipocytes reported here are present in precursor cells. However, it has been reported that DMS between lean and obese are largely maintained between human adipose derived stem cells and mature adipocytes [48]. The impaired lipid storage observed after candidate gene knockdown could thus be the result of impaired adipogenesis.

5. CONCLUSION

In conclusion, results presented herein support the notion that differential CpG-methylation in adipocytes is linked to hypertrophic WAT morphology predisposing to T2D. Several genes which appear to be under epigenetic control and influence adipocyte lipid storage and metabolism were identified.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

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