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ARTICLE

Epigenetic programming of adipose-derived stem cells in low birthweight individuals

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Abstract

Aims/hypothesis Low birthweight (LBW) is associated with dysfunctions of adipose tissue and metabolic disease in adult life. We hypothesised that altered epigenetic and transcriptional regulation of adipose-derived stem cells (ADSCs) could play a role in programming adipose tissue dysfunction in LBW individuals.

Methods ADSCs were isolated from the subcutaneous adipose tissue of 13 normal birthweight (NBW) and 13 LBW adult men. The adipocytes were cultured in vitro, and genome-wide differences in RNA expression and DNA methylation profiles were analysed in ADSCs and differentiated adipocytes.

Results We demonstrated that ADSCs from LBW individuals exhibit multiple expression changes as well as genome-wide alterations in methylation pattern. Reduced expression of the transcription factor cyclin T2 encoded by *CCNT2* may play a key role in orchestrating several of the gene expression changes in ADSCs from LBW individuals. Indeed, silencing of *CCNT2* in human adipocytes decreased leptin secretion as well as the mRNA expression of several genes involved in adipogenesis, including *MGLL*, *LIPE*, *PPARG*, *LEP* and *ADIPOQ*. Only subtle genome-wide mRNA expression and DNA methylation changes were seen in mature cultured adipocytes from LBW individuals.

Conclusions/interpretation Epigenetic and transcriptional changes in LBW individuals are most pronounced in immature ADSCs that in turn may programme physiological characteristics of the mature adipocytes that influence the risk of metabolic diseases. Reduced expression of *CCNT2* may play a key role in the developmental programming of adipose tissue.

Keywords Adipocytes · Adipogenesis · Adipose tissue · Cyclin T2 · Epigenetics · Fetal programming · Low birthweight · Metabolic disease · Type 2 diabetes

Abbreviations

ADSC	Adipose-derived stem cells
CCNT2	Cyclin T2
CDK	Cyclin-dependent kinase
FDR	False discovery rate
GO	Gene ontology
LBW	Low birthweight
NBW	Normal birthweight
PCA	Principal component analyses
RT	Reverse transcription
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STAT2	Signal transducer and activator of transcription 2

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Introduction

Environmental factors leading to inadequate intrauterine nutrition or decreased placental effectiveness can result in low birthweight (LBW) [1]. In adulthood, individuals born with LBW have different phenotypic changes including reduced lean body mass [2], higher total abdominal fat mass [3], reduced insulin secretion [4] and altered expression of insulin signalling proteins in muscle and fat [5, 6]. Together, these changes are key determinants of an increased risk of developing cardiometabolic diseases such as type 2 diabetes [7].

Epigenetic processes are conceptually attractive mechanisms explaining how events occurring in fetal life can permanently affect tissue structure and metabolism, and thereby mediate the association between LBW and the development of type 2 diabetes in adulthood. DNA methylation patterns are copied by DNA methyltransferase 1 (DNMT1) during DNA replication [8], implying that environmentally induced perturbations that occur during early development can be transmitted through cell divisions to subsequent cell generations. Alterations in the fetal environment may therefore change the epigenome of specific cells or tissues, thereby permanently changing the structure and/or function of different organs [9–12]. Interestingly, exposure to suboptimal nutrition during fetal life leads to changes in germ cell DNA methylome in male mice [13], potentially affecting even the next generation. In addition, maternal diet during early pregnancy in humans can cause persistent changes in the blood methylome of the offspring [14].

Adipose-derived stem cells (ADSCs) originate from the mesenchymal lineage and are formed in the second trimester of pregnancy [15]. ADSCs serve to generate and regenerate adult adipose tissue through differentiation into lipid-storing and metabolically active adipocytes [16], a process that is controlled by epigenetic mechanisms [17–20]. Human ADSCs can be isolated with high purity from the stromal vascular fraction of subcutaneous fat biopsies. Importantly, compared with many other cellular lineages, the process of fat cell differentiation in vitro is considered to be fairly authentic, recapitulating most of the key features of adipogenesis in vivo, including morphology, cessation of cell growth, expression of lipogenic enzymes, sensitivity to lipogenic hormones and extensive lipid accumulation [21].

We recently demonstrated that cultured pre-adipocytes from LBW humans exhibit several functional defects associated with adipogenesis, including impaired leptin and adiponectin production and decreased expression of the genes encoding peroxisome proliferator-activated receptor gamma (*PPARG*) and fatty acid binding protein 4 (*FABP4*), together suggesting functional immaturity of the cells. The impaired leptin production was associated with increased DNA methylation of the *LEP* promoter [22]. Based on these compelling observations, we hypothesised that LBW individuals would exhibit disproportionately more widespread DNA methylation and gene expression changes in the ADSCs, and that the methylome and transcriptome of ADSCs might be an indicator of the early-life environment potentially influencing the risk of developing cardiometabolic diseases with ageing.

Methods

Study participants Thirteen individuals born with LBW (birthweight below the 10th percentile) and 13 individuals born with normal birthweight (NBW) (birthweight in the 50th–90th percentile range) were recruited through The Danish National Birth Register. Briefly, all individuals were healthy, young, age-matched male singletons, born at term, with no family history of diabetes in two generations, with a BMI < 30 kg/m^2 and without self-reported high physical activity level (>10 h per week). Abdominal subcutaneous adipose biopsies were obtained from the individuals using a Bergstrom biopsy needle with suction [23] after an overnight fast. Five individuals participated in a study by Jørgensen et al [24] and 21 individuals in a study by Mortensen et al [25], both of which used the same stringent inclusion criteria. The number of participants included in this study is lower than the total number of participants involved in the studies by Jørgensen and Mortensen [24, 25] only because ADSCs were not available for all participants.

Study approval All participants were given oral and written information about the experimental procedure before giving their written informed consent. The study was approved by the regional ethical committee (H-A-2009-040 and H-D-2008-127) and performed according to the Declaration of Helsinki. In all data analyses, participants were identified by number rather than by name.

Isolation and culture of adipose precursor cells Adipose precursor cells were isolated from subcutaneous biopsies. A detailed description of the cell isolation and culture procedures is given in the electronic supplementary material [ESM] Methods.

Small interfering RNA transfection in adipocytes Transient transfections of human adipocytes were performed using pools of small interfering RNA (siRNA) oligos specifically targeting four different sites of *STAT2* or *CCNT2* mRNA (On Target Plus) to minimise any off-target effects (catalogue no. L-003221-00-0010 and L-012064-00-0010 10, Dharmacon, CO, USA). Control cells were treated with a commercial non-targeting siRNA control (D-001810-10-20; Dharmacon). Transfections were performed in differentiating adipocytes at

day 6 using 50 nmol/l of siRNA and Lipofectamine RNAiMAX (catalogue no. 13778-075, Invitrogen, Carlsbad, CA, USA) in antibiotic-free differentiation media (see ESM). RNA and media were harvested at day 9 (72 h after transfection) and at day 12 (144 h after transfection). Intracellular lipid droplets were stained with Oil Red O at day 12 as previously described [22]. Leptin concentrations were measured in cell media at day 12 using Meso Scale Discovery plates (catalogue no. K151BYC-1; MSD, MD, USA) according to the manufacturer's instructions (without diluting the media samples).

Gene expression and DNA methylation arrays Genome-wide gene expression analysis was studied using the HumanHT-12 v4 Expression BeadChip (Illumina, CA, USA). Genome-wide DNA methylation was studied using the Infinium HumanMethylation450 BeadChip (Illumina). A detailed description of data processing as well as gene ontology (GO) analysis and molecular interaction networks is given in ESM Methods.

cDNA synthesis and quantitative real-time PCR Reverse transcription (RT) reactions were performed using random hexamers on 0.25 μg RNA employing the High-Capacity cDNA RT kit (Applied Biosystems, CA, USA). Primers were designed using human specific databases (Ensembl) and the Universal Probe Library Assay Design Centre (https://lifescience.roche.com, accessed July 2016). The primer sequences are given in ESM Table 1. The primers were synthesised by DNA Technology (Risskov, Denmark). The real-time PCR was performed using the SYBR green method with quantification by standard curves on a ViiA 7 Real-Time PCR System (Applied Biosystems). To adjust for variations in the amount of cDNA, each gene expression quantity was normalised to that of 18S ribosomal RNA.

Statistical analysis Results are presented as mean ± SD, mean ± SEM, mean differences and/or fold change. Differences in gene expression and DNA methylation between the LBW and NBW groups in ADSCs and mature adipocytes were analysed using non-paired, non-parametric tests (Wilcoxon rank-sum tests). The false discovery rate (FDR) [26] was applied to account for multiple testing in all the genome-wide analyses of gene expression and DNA methylation performed. The R project (www.r-project.org/; accessed February 2012) was used for statistical analyses and graphics, with additional packages provided by the Bioconductor project (www.bioconductor.org/; accessed February 2012) [27]. We performed a principal component analysis (PCA) of the genome-wide DNA methylation and gene expression data after batch correction and correlated the top principal components with birthweight, age and BMI to examine possible sources of variation in the data sets. The data used for PCA were based on uncorrected p values. We used two-way ANOVA (SAS Software, version 9.1.3 (SAS Institute, Cary, NC, USA) to evaluate the effect of differentiation and group on *STAT2* and *CCNT2* expression. The residuals obtained from the ANOVA models were evaluated, and the model was accepted only if the residuals were normally distributed.

Results

Clinical characteristics of the human donors ADSCs were isolated from subcutaneous biopsies obtained from 13 NBW and 13 LBW adult lean healthy men. The clinical characteristics of the ADSC donors are shown in Table 1. There was no significant difference in fasting serum insulin or plasma glucose (Table 1), or plasma triacylglycerol and cholesterol levels (data not shown) between LBW and NBW individuals ($p > 0.05$). However, the LBW men were lighter ($p < 0.05$) and had a tendency towards an increased WHR ($p = 0.058$), indicating a higher proportion of abdominal fat relative to hip fat. Genome-wide gene expression and DNA methylation levels were analysed in proliferating ADSCs (80% confluent) and terminally differentiated adipocytes grown for 12 days in differentiation media. Statistical comparisons are indicated by the black arrows in Fig. 1a. The previously described phenotype of the LBW adipocytes includes impaired secretion of adiponectin and leptin together with decreased gene expression of *FABP4* and *PPARG* [22]. Indeed, the same donors and cell cultures were used in the current study.

Genome-wide effects of birthweight on gene expression and DNA methylation To explore the effect of birthweight on genome-wide gene expression and DNA methylation,

Table 1 Clinical characteristic of ADSC donors

	LBW ($n = 13$)	NBW ($n = 13$)	p value
Birthweight (kg)	2.7 ± 0.1	3.7 ± 0.2	<0.0001
Age (years)	22.4 ± 1.7	23.2 ± 1.6	NS
Weight (kg)	73.9 ± 7.3	80.8 ± 5.4	<0.05
Height (cm)	178.0 ± 4.1	182.5 ± 6.9	0.063
BMI (kg/m^2)	23.3 ± 2.2	24.3 ± 2.0	NS
WHR	0.90 ± 0.1	0.86 ± 0.1	0.058
Total fat mass (kg)	13.2 ± 4.0	13.1 ± 2.8	NS
Trunk fat mass (%)	16.6 ± 4.7	15.4 ± 3.5	NS
Lean body mass (kg)	58.5 ± 4.5	64.4 ± 4.4	<0.01
Fasting insulin (pmol/l)	28.8 ± 17.4	37.3 ± 20.5	NS
Fasting glucose (mmol/l)	4.9 ± 0.4	5.1 ± 0.2	NS
Fasting HbA_{1c} (%)	5.2 ± 0.3	5.1 ± 0.2	NS
Fasting HbA_{1c} (mmol/mol)	32.8 ± 3.1	32.2 ± 2.5	NS

Data are shown as mean ± SD

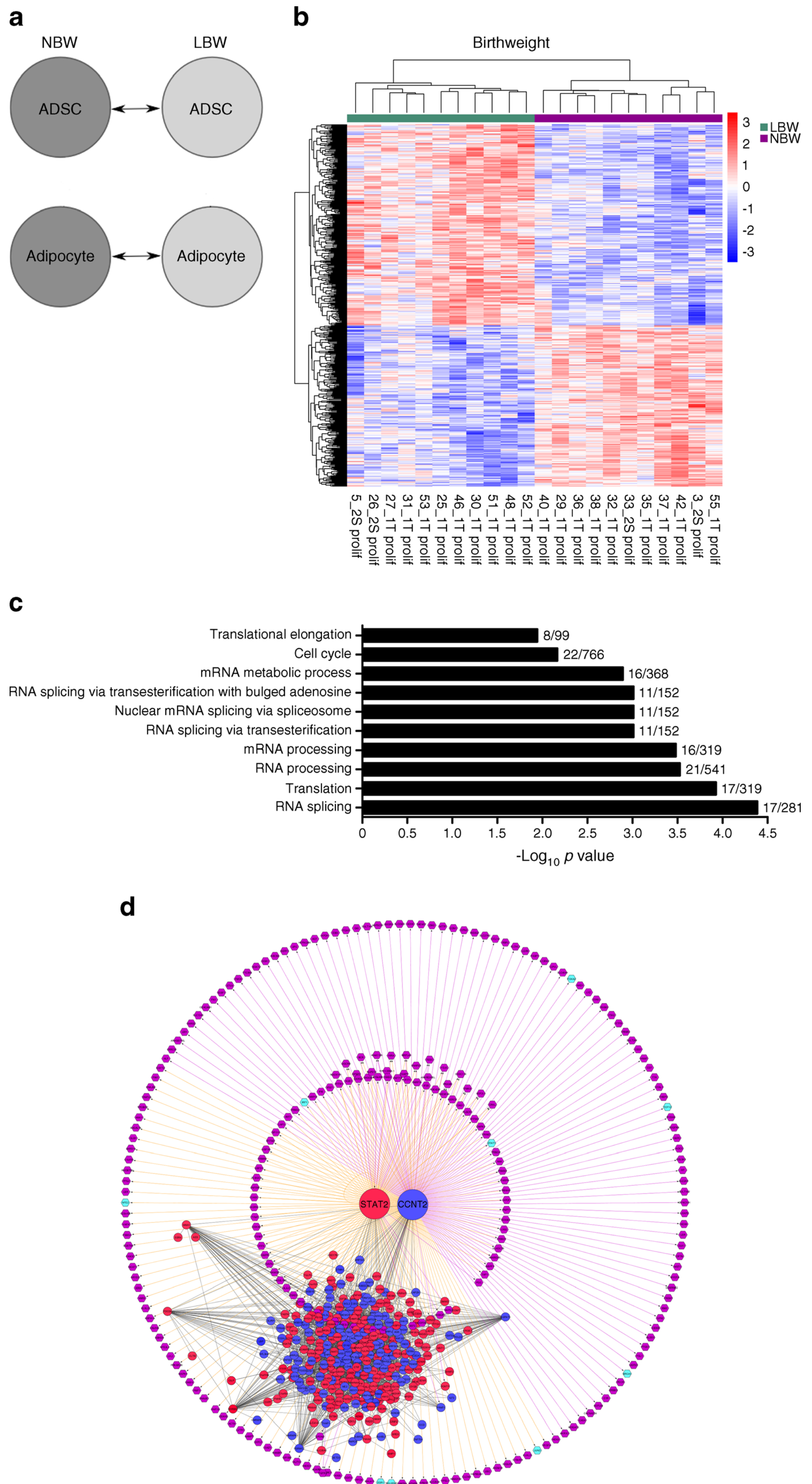

Fig. 1 Gene expression differences between ADSCs from LBW and NBW donors. (**a**) Study set-up. Genome-wide gene expression and DNA methylation profiles were analysed in ADSCs and mature adipocytes from NBW individuals and LBW individuals. Comparisons between sample groups were performed as indicated by the black arrows. (**b**) Hierarchical clustering heat map of 506 differentially expressed mRNA transcripts ($q < 0.05$) in ADSCs isolated from LBW vs NBW individuals. Each column represents ADSCs isolated from a single sample, and each row represents a single mRNA transcript. Expression levels are scaled to raw z scores and plotted in a red to blue colour scale representing high to low expression signals. Hierarchical clustering was applied based on Euclidean distances. (**c**) GO biological processes based on 225 downregulated gene transcripts in LBW ADSCs. The number of significant genes out of all genes in the GO biological process is shown at the end of the bar. The p values are Benjamini–Hochberg corrected. (**d**) GeneMANIA network illustrating genetic interactions (black lines) between genes showing significantly ($q < 0.05$) higher expression (red nodes) or lower expression (blue nodes) in ADSCs from LBW cells. To look for transcription factors (TFs) among the differentially expressed genes, the network is extended with TF targets from the ENCODE project. TF–gene or TF–TF interactions are indicated in the extended network as proximal (yellow lines) or distal (purple lines) TF regulation. TF targets can be assigned either as a significant differentially expressed gene in our data set (red to blue nodes), or any other gene (purple nodes) or TF (turquoise nodes) not showing differential expression in ADSCs between the two birthweight groups. Magnified genes (*CCNT2* and *STAT2*) are TFs with a central role in the gene interaction network

PCAs were performed first within the group of ADSCs, and second within the group of differentiated adipocytes. A significant correlation was observed between the second principal component and birthweight when analysing gene expression profiles in ADSCs from the two groups ($p = 0.0234$), whereas there was no correlation between birthweight and principal components in the differentiated adipocytes. Remarkably, a similar and even more pronounced pattern was reflected in the genome-wide methylation data, where a significant correlation was observed between the first principal component and birthweight in ADSCs ($p = 0.0231$). Again, there was no effect of birthweight on the methylation pattern in the differentiated adipocytes, suggesting that both gene expression and DNA methylation changes in LBW participants are most pronounced in immature tissue progenitor cells.

Identification of *CCNT2* and *STAT2* mRNA as differentially expressed transcription factors in LBW ADSCs At an individual gene level, 506 Illumina-assigned gene transcripts were significantly differentially expressed at an FDR of less than 5% ($q < 0.05$) when comparing ADSCs from the two birthweight groups (Fig. 1b, ESM Table 2). Of the differently expressed genes, 281 transcripts were upregulated in LBW ADSCs, and 225 transcripts were downregulated in LBW ADSCs. When subjecting genes downregulated in ADSCs derived from LBW individuals to GO analysis, significant enrichment of genes in ten biological processes was found. The biological processes reflected altered function of the ribosome and spliceosome, and the levels of several genes important for cell cycle progression were decreased (Fig. 1c). No significant biological processes were obtained after subjecting the upregulated transcripts in LBW ADSCs to GO analysis. To identify central transcription factors, which could drive the altered gene expression in LBW ADSCs, information from the ENCODE project (www.genome.gov/10005107/encode-project/, accessed January 2015) was applied to a gene–gene interaction network build upon genes both upregulated and downregulated in LBW ADSCs. Two differentially expressed transcription factors cyclin T2 (*CCNT2*) (downregulated in LBW) and signal transducer and activator of transcription 2 (*STAT2*) (upregulated in LBW) were shown to interact with a large number of genes also significantly differentially expressed in our data set (red and blue nodes) (Fig. 1d). The analysis suggested that CCNT2 and STAT2 could be central to the gene expression changes in LBW ADSCs by potentially regulating the gene expression levels, and we therefore decided to investigate *CCNT2* and *STAT2* in more detail. *STAT2* expression increased with differentiation (ANOVA; $p < 0.0001$) and *CCNT2* expression decreased with differentiation (ANOVA, $p < 0.01$). In addition, both *STAT2* and *CCNT2* expression levels were

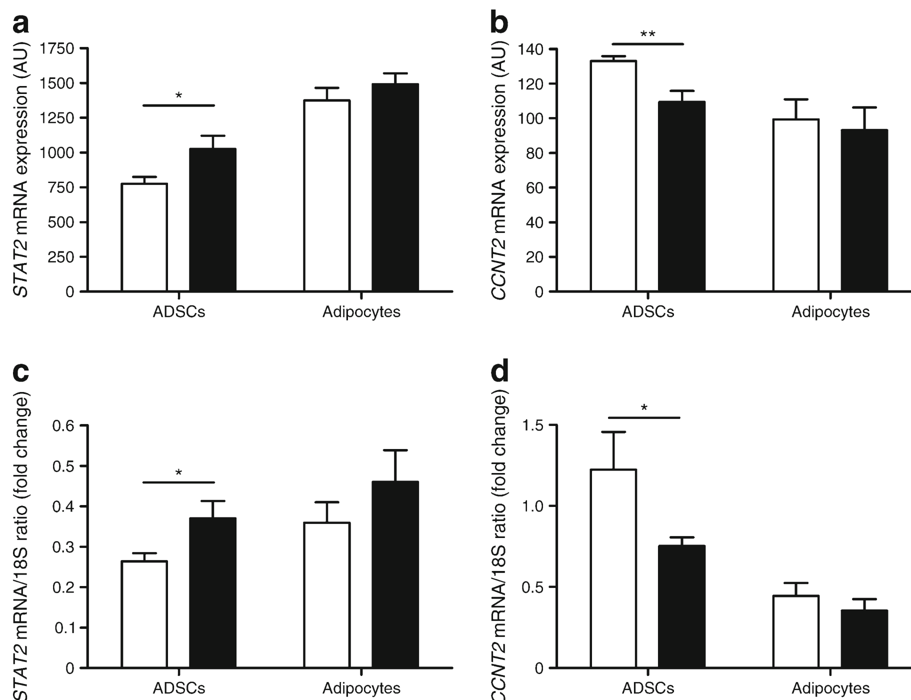

Fig. 2 (**a**, **b**) Gene expression of *STAT2* and *CCNT2* in ADSCs and adipocytes. Illumina mRNA expression of *STAT2* (**a**) and *CCNT2* (**b**) in ADSCs and differentiated adipocytes. White bars, NBW; black bars, LBW. Whereas $\log_2$ intensity values after robust multiarray average data processing and normalisation were used for all statistical tests, unlogged expression values are plotted in the figure, in arbitrary units (AU). Data are shown as mean ± SEM. (**c**, **d**) Validation of *STAT2* (**c**) and *CCNT2* (**d**) mRNA expression in ADSCs and differentiated adipocytes from NBW ($n = 13$) and LBW ($n = 13$) individuals using real-time PCR. The mRNA levels were normalised to 18S ribosomal RNA. White bars, NBW; black bars, LBW. Data are shown as mean ± SEM. The effect of differentiation was estimated using two-way ANOVA. *$p < 0.05$, **$p < 0.01$

different between the birthweight groups in ADCSs but not in adipocytes (Fig. 2a, b). The differential expression was technically validated with quantitative real-time PCR, which confirmed the birthweight-related differences at the ADSC stage (Fig. 2c, d).

We found no individual CpG site with differential DNA methylation after FDR correction ($q < 0.05$) when comparing ADSCs between birthweight groups (ESM Table 3). The 20 most significant CpG sites with differential methylation between LBW and NBW ADSCs without correction for multiple testing are shown in Table 2. There was no overlap between the 20 most significant CpG sites and the 506 differentially expressed transcripts between NBW and LBW ADSCs. Taken together, these findings suggest that inherent DNA methylation changes in LBW ADCSs do not directly explain the birthweight-dependent alterations in *CCNT2* and *STAT2* expression.

***CCNT2* and *STAT2* play a role in human adipogenesis** To further investigate the role of CCNT2 and STAT2 in adipogenesis, we used siRNA to silence *CCNT2* and *STAT2* during early adipogenesis (day 6) in cells from five different NBW donors. Both *STAT2* and *CCNT2* mRNA levels were efficiently decreased after 3 days (at day 9) and after 6 days (day 12) of silencing (Fig. 3a, b). Although no differences were observed for fat accumulation (Fig. 3c), a striking decrease in the gene expression of five out of six a priori selected adipogenic genes was observed (Fig. 3d) after silencing of *CCNT2*. Four of the genes (*PPARG*, *FABP4*, *LEP* and *ADIPOQ*) were selected based on previously reported decreased expression in LBW adipocytes [22], whereas *MGLL* (encoding monoglycerol lipase) and *LIPE* (encoding hormone-sensitive lipase) were selected on the basis of their important role in lipolysis. *CCNT2* silencing reduced the expression of *MGLL*, *LIPE*, *PPARG*, *LEP* and *ADIPOQ* by approximately 50% compared with control cells, whereas *STAT2* silencing reduced the expression of *FABP4* by more than 50%. We further investigated the secretion of leptin in the media of mature adipocytes. After siRNA silencing of *STAT2*, leptin was not significantly altered ($p = 0.1$) (Fig. 3e); however, a modest but significant decrease was observed after siRNA silencing of *CCNT2* (Fig. 3f).

We further sought to investigate whether single nucleotide polymorphisms (SNPs) in the *STAT2* (12q13.3) or the *CCNT2* (2q21.3) gene regions (NCBI Variation Viewer, www.ncbi.nlm.nih.gov/variation/view; accessed July 2016) were associated with cardiometabolic disease or related quantitative traits using the publicly available genome-wide association study meta-analysis results from the DIAGRAM (DIAbetes Genetics Replication And Meta-analysis) [28], CARDIoGRAM (Coronary ARtery DIsease Genome wide

Table 2 Methylation differences between NBW and LBW ADSCs

Gene name	Probe ID	p values	q values	Difference (NBW minus LBW) (%)
n/a	cg14459772	3.85×10^{-7}	0.17	−7.5
SHANK2	cg20170028	1.35×10^{-6}	0.20	−1.3
LY6H	cg09528449	2.31×10^{-6}	0.20	2.9
SNX14	cg13188409	3.65×10^{-6}	0.20	1.2
SLC16A11	cg18530716	3.65×10^{-6}	0.20	−2.0
NCLN	cg20667124	3.65×10^{-6}	0.20	−2.8
AAAS	cg23032316	3.65×10^{-6}	0.20	−4.3
CARD11	cg26937500	3.65×10^{-6}	0.20	−1.7
DMAP1	cg03726147	5.77×10^{-6}	0.21	−6.7
CEACAM19	cg10143883	5.77×10^{-6}	0.21	−3.7
EIF4A3	cg10503854	5.77×10^{-6}	0.21	−0.3
DYNLT1	cg13598865	5.77×10^{-6}	0.21	0.6
CSK	cg00516515	8.65×10^{-6}	0.21	−4.3
FAM120B	cg01956781	8.65×10^{-6}	0.21	0.7
n/a	cg04594598	8.65×10^{-6}	0.21	2.6
n/a	cg05759166	8.65×10^{-6}	0.21	−7.0
BICC1	cg22641201	8.65×10^{-6}	0.21	1.5
ACOX3	cg22777162	8.65×10^{-6}	0.21	−2.2
C2CD2L	cg03947203	1.29×10^{-5}	0.23	−9.7
TRIM11	cg04632980	1.29×10^{-5}	0.23	−5.8

The 20 CpG sites with the lowest p values for the methylation difference between the LBW and NBW groups are shown for ADSCs. Each CpG site is identified with its Illumina probe target ID. The difference in methylation percentage is shown as calculated by methylation in the NBW group minus methylation in the LBW group

n/a, this probe has not been associated with any gene by Illumina

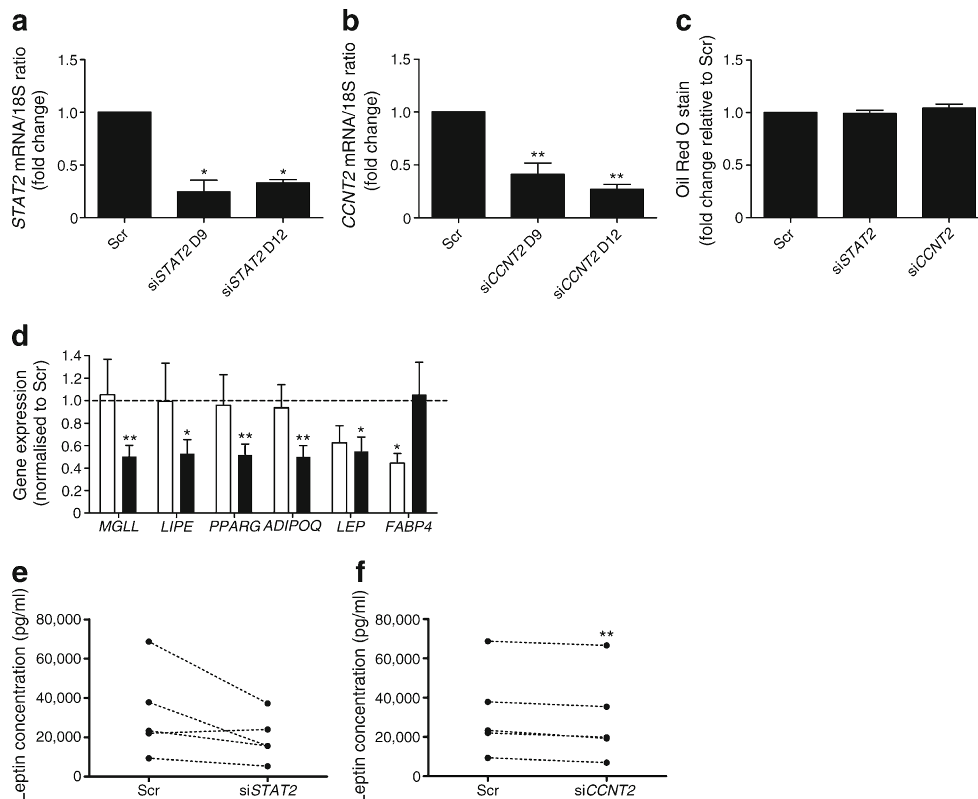

Fig. 3 Effect of STAT2 and CCNT2 on adipogenesis. (**a**, **b**) siRNA silencing of *STAT2* (**a**) and *CCNT2* (**b**) was performed during early differentiation (day 6) in cells isolated from five different individuals (NBW) (Scr, scrambled). Efficient knockdown of both transcripts was observed at day 9 and day 12. The mRNA levels were normalised to 18S ribosomal RNA ($n = 5$). (**c**) Oil Red O incorporation was estimated in mature adipocytes. (**d**) The mRNA levels of a selected panel of adipogenic markers were investigated in mature adipocytes. The mRNA levels were normalised to 18S ribosomal RNA. White bars, si*STAT2*, black bars, si*CCNT2*. In (**a**–**d**), paired *t* tests were performed on raw values, whereas the figures show the ratio of the knockdown in relation to Scr (to visualise the effect of siRNA silencing as a fold change). (**e**, **f**) The amount of leptin in the media from mature adipocytes (day 12) was analysed by ELISA. $^*p < 0.05$, $^{**}p < 0.01$

Replication and Meta-analysis) [29], MAGIC (Meta-Analyses of Glucose and Insulin-related traits Consortium) [30–32] and GIANT (Genetic Investigation of ANthropometric Traits) [33, 34] consortia. The traits examined were type 2 diabetes (case–control), coronary artery disease (case–control), fasting glucose, fasting insulin, 2 h glucose, HOMA-IR, BMI and WHR. The findings were reported with the original consortia *p* values, and in order to correct for the multiple look-ups, we calculated a Bonferroni significance threshold as $p < 0.05$/number of SNPs examined/number of traits examined. Twenty-nine SNPs were examined near *STAT2* and 86 near *CCNT2*, giving a Bonferroni threshold of approximately $p < 7 \times 10^{-5}$ to $p < 2 \times 10^{-4}$ depending on the gene region. Strikingly, we found that the majority of SNPs examined in the *CCNT2* region (2q21.3) were associated with BMI (59 of 85 SNPs), and one of these associations (rs1530559, $p = 2 \times 10^{-6}$) remained significant after correction for multiple comparisons. In addition, 31 of 83 SNPs in the *CCNT2* region were associated with HOMA-IR, although not significantly after correction for multiple comparisons (ESM Table 4; see ESM Table 4 legend for details). In the gene region surrounding *STAT2*, only a few SNPs associated nominally with the traits examined. Most interesting was the finding of four SNPs associating with coronary artery disease ($p < 0.05$) out of the eight SNPs examined in this meta-analysis (ESM Table 5; see ESM Table 5 legend for details).

A lack of genome-wide DNA methylation and gene expression changes in mature adipocytes We found no gene expression probes with altered expression or individual CpG sites with differential DNA methylation when comparing mature adipocytes between birthweight groups, after correction for multiple testing (ESM Tables 6 and 7). The 20 most significant CpG sites with differential methylation between LBW and NBW adipocytes without correction for multiple testing are shown in Table 3.

Discussion

We demonstrated that adipogenic progenitor cells from the abdominal subcutaneous fat depot of LBW individuals have an altered expression of 506 transcripts, including increased expression of *STAT2* and decreased expression of *CCNT2*. Silencing of *CCNT2* changed the expression of five selected

Table 3 Methylation differences between NBW and LBW adipocytes

Gene name	Probe ID	*p* values	*q* values	Difference (NBW minus LBW) (%)
ARHGEF18	cg01977519	2.31×10^{-6}	0.26	1.5
FAM81B	cg23360388	2.31×10^{-6}	0.26	3.6
OXCT1	cg21515384	3.65×10^{-6}	0.26	−2.8
MCCC1	cg23476885	3.65×10^{-6}	0.26	−2.9
n/a	cg04568823	5.77×10^{-6}	0.26	12.7
n/a	cg04850254	5.77×10^{-6}	0.26	2.7
SMAD9	cg10436026	5.77×10^{-6}	0.26	−8.4
SPATA20	cg19587434	5.77×10^{-6}	0.26	1.5
MGRN1	cg01922891	8.65×10^{-6}	0.26	−10.2
WNT5B	cg05175896	8.65×10^{-6}	0.26	−12.1
NUP210	cg08091398	8.65×10^{-6}	0.26	10.2
PCSK2	cg15060366	8.65×10^{-6}	0.26	3.3
ZAP70	cg18005337	8.65×10^{-6}	0.26	−14.1
NLRC4	cg23459416	8.65×10^{-6}	0.26	3.8
n/a	cg27422722	8.65×10^{-6}	0.26	−1.4
RILP	cg04313875	1.29×10^{-5}	0.26	−1.0
VAX2	cg07094440	1.29×10^{-5}	0.26	3.7
n/a	cg07617283	1.29×10^{-5}	0.26	10.9
SH3BP4	cg13858407	1.29×10^{-5}	0.26	−11.5
C9orf40	cg19102271	1.29×10^{-5}	0.26	−1.7

The 20 CpG sites with the lowest *p* values for the methylation difference between the LBW and NBW groups are shown for adipocytes. Each CpG site is identified with its Illumina probe target ID. The difference in methylation percentage is shown as calculated by methylation in the NBW group minus methylation in the LBW group

n/a, this probe has not been associated with any gene by Illumina

genes, which promote adipogenesis. CCNT2 might therefore play a role in the developmental programming of adipose tissue dysfunction in LBW humans.

Distinct epigenetic changes have been identified in the muscle and fat tissues of LBW individuals using a candidate gene approach [9, 35], and altered genome-wide changes in DNA methylation in the muscle tissue of LBW individuals have been observed following a metabolic challenge such as overfeeding [36]. Nonetheless, the epigenetic changes reported in mature tissue (biopsies) from LBW individuals have so far been relatively modest and cannot alone explain the strong association between LBW and the development of type 2 diabetes [7]. We hypothesised that epigenetic changes resulting from an unfavourable intrauterine environment causing LBW would be detectable and potentially more pronounced in tissue progenitor cells, e.g. ADSCs from adipose tissue. In support of our hypothesis, we here provide evidence that large parts of the variation in the gene expression and DNA methylation data set are explained by birthweight, reflected by significant correlations between the second principal component and birthweight in the gene expression data, and between the first principal component and birthweight in the DNA methylation data. Importantly, after ADSCs had differentiated into mature adipocytes, the grouping by birthweight disappeared. The finding of a clearer separation of the epigenetic fingerprints between LBW and NBW individuals in the less differentiated cells support our a priori hypothesis that epigenetic changes associated with fetal programming are most pronounced, and therefore may also be more functionally important in the immature progenitor cells.

The consequences of an altered function of ADSCs could be fewer and larger adipocytes, but it could also be the production of less developed adipocytes with an altered capacity to store fat and regulate lipolysis. Indeed, young men with LBW have increased whole-body basal lipolysis [37] as well as a significantly higher proportion of abdominal fat mass relative to total fat mass [3]. Importantly, such changes are suspected to influence the risk of developing cardiometabolic diseases [38]. The lack of genome-wide findings in mature cultured adipocytes might be explained by methodological limitations as adipocytes derived by in vitro differentiation of ADSCs will be affected by the strong adipogenic cocktail with maximal inhibition of, for example, lipolysis due to the high media contents of insulin, rosiglitazone and dexamethasone; it therefore cannot truly account for the changes that could have occurred in adipocytes in vivo.

The genome-wide transcriptomic disparities between LBW and NBW ADSCs seemed to centre on an altered regulation of the transcription factor *CCNT2* (downregulated in LBW

ADSCs). CCNT2 is the regulatory subunit of the cyclin-dependent kinase (CDK) pair (CDK9–CCNT2), which facilitates the transition from abortive to productive elongation by phosphorylating the large subunit of RNA polymerase II [39]. CCNT2 is essential for mouse embryogenesis [40] and is involved in early spermatogenesis [41], suggesting that CCNT2 is active during the earliest developmental stages. We found that silencing of *CCNT2* markedly reduced the expression of a panel of carefully selected adipogenic markers, suggesting that CCNT2 is a central regulator of adipocyte development. In support of an important role of the CCNT2–CDK9 complex in adipogenesis, it was shown that overexpression of CDK9 in 3T3L1 adipocytes increased adipogenesis by direct binding to and phosphorylation of proliferator-activated receptor gamma [42]. We previously reported a reduced expression of *PPARG*, *ADIPOQ* and *LEP* in cultured adipocytes derived from LBW individuals [22]. Importantly, these are the same markers that we find decreased in the current study after *CCNT2* silencing. In addition, a marked decrease of the central enzymes controlling lipolysis, *MGL* and *LIPE*, were seen after *CCNT2* silencing. Altogether, reduced expression of *CCNT2* in LBW ADSCs might lead to delayed initiation of the adipogenic programme and subsequent development of immature adipocytes.

We next sought to investigate whether SNPs in the *CCNT2* gene region (2q21.3) were associated with cardiometabolic disease and related quantitative traits in previously published genome-wide association study meta-analyses. These look-ups showed that genetic variation of the *CCNT2* gene region was associated with obesity and insulin resistance, providing further evidence for a functional role of *CCNT2* in adipose tissue. Indeed, one SNP in the proximity of *CCNT2*, *MAP3K19* rs1530559, associated with BMI at the suggestive genome-wide significance threshold ($p<5\times10^{-6}$) [33] and also relatively strongly with fasting insulin and HOMA-IR [30].

We initially hypothesised that both epigenetic and transcriptional regulation of ADSCs could play a role in programming adipose tissue dysfunctions in LBW individuals. Although in the genome-wide DNA methylation data from ADSCs, we found a significant correlation between the first principal component and birthweight, we did not identify any single CpG site with differential methylation between NBW and LBW ADSCs when correcting for multiple testing. This could suggest that several small changes are operating together to change gene expression in LBW ADSCs. However, this finding also allows for the possible influence of histone modifications or changes in miRNA expression as causal factors regulating the 506 differentially expressed transcripts, including *CCNT2* and *STAT2*, in LBW ADSCs. Importantly, the biopsies were obtained in adult men, and although the groups were well-matched according to current habits, e.g. time spent training and smoking habits, as well as according to BMI, sex and age, confounding factors including environmental stimuli might have accumulated during life and could affect the methylation and expression levels.

In conclusion, we show that ADSCs derived from healthy adult LBW humans with increased risk of developing type 2 diabetes due to their lower birthweight exhibit widespread genome-wide transcription as well as DNA methylation profile changes. This provides further evidence that inadequate fetal energy supply permanently changes the epigenetic regulation of adipose tissue progenitor cells. Further research is needed to understand whether or how these findings may be translated into a more sustainable intergenerational prevention of type 2 diabetes and associated cardiometabolic diseases in future generations.

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Contribution statement CB, AHO, AV, CS and CL conceived and designed the study, analysed the data, and wrote the paper. CB, KSS, MS and NSH carried out cell studies. AP, AHO and RR-M conducted the bioinformatics analyses. SWJ and BM collected the human cohorts and analysed and interpreted the human clinical data. All co-authors revised the manuscript and approved the final version. CB is responsible for the integrity of the work as a whole.

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