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CD24 is required for regulating gene expression, but not glucose uptake, during adipogenesis

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ABSTRACT

Adipose tissue dysfunction in obesity and lipodystrophy results in major health complications such as heart disease and stroke, and is associated with an increased risk of some cancers. We have previously found that the cell surface receptor CD24 regulates adipogenesis as measured by lipid accumulation and gene expression in mature adipocytes. How CD24 regulates these processes remains unknown. To begin answering this question, we first determined that CD24 does not affect glucose uptake in differentiating adipocytes *in vitro*. We then examined changes in global gene expression via DNA microarray in 3T3-L1 adipocytes with siRNA-mediated knock-down of CD24 expression. We found that CD24 expression is necessary for upregulation of up to 134 genes. We validated the CD24-mediated regulation of 4 of these genes during *in vitro* adipogenesis of 3T3-L1 and primary cells isolated from the inguinal white adipose tissue depots of CD24 knockout mice. Surprisingly, we found that only 1 of these genes was also regulated by CD24 in cells from the epididymal depot. Overall, these data suggest that CD24 is necessary for select gene expression in a depot-specific manner during adipogenesis *in vitro*. These findings could help elucidate the mechanisms regulating lipid accumulation in adipocytes thereby aiding in the development of novel treatment strategies for obesity and lipodystophy.

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Introduction

White adipose tissue (WAT) stores energy in the form of triglycerides (TGs) and cholesterol esters along with being an essential endocrine organ.[1] WAT is dispersed throughout the body in subcutaneous WAT depots that are present below the skin and in visceral WAT depots that surround the internal organs. Subcutaneous WAT and visceral WAT depots have numerous differences in metabolic characteristics,[2,3] response to diet,[4] gene expression patterns,[5,6] and developmental origins.[7–9]

WAT consists of a heterogeneous population of cells that includes adipose derived stem cells (ADSCs), pre-adipocytes and mature adipocytes as well as macrophages/monocytes and other immune cells, fibroblasts, and progenitor endothelial cells, among others.[10,11] Pre-adipocytes and ADSCs undergo adipogenesis to differentiate into mature adipocytes. Adipogenesis is tightly controlled through activation of the master regulators of adipogenesis, CCAAT/enhancer-binding proteinα *(C/EBPα)* and peroxisome proliferator-activated receptor γ (*Pparγ*), which play important roles in further activating multiple downstream genes for adipogenesis.[10] In addition, the decreased expression of negative regulators of adipogenesis, such as Pref-1, is required for full differentiation of adipocytes.[12,13]

WAT grows by hypertrophy, an increase in cell size due to lipid accumulation, or by hyperplasia, an increase in cell number, depending on the age of the invidual.[14,15] Lipid accumulation in adipocytes is achieved by either *de novo* lipid synthesis (lipogenesis) or via the uptake of existing free fatty acids (FFA) *in vivo*. Glucose acts as a lipogenic precursor and leads to the production of acetyl-Coenzyme A (CoA) which can then be used to synthesize FFA as part of *de novo* TG synthesis. *In vivo* 20–25% of all the glucose taken up by adipocytes is incorporated into TG.[16]

CD24, a heavily glycosylated GPI-linked cell surface receptor, is a marker of pre-adipocytes *in vivo*[17,18] as well as a specific regulator of adipogenesis *in vitro*.[19] Pre-adipocytes that express CD24 are capable of reconstituting fully functional WAT depots *in vivo* in lipodystrophic mice.[17] In addition, mice fed a high-fat diet were found to rapidly gain and then lose CD24-expressing adipocyte progenitor cells.[20] CD24 mRNA and surface protein expression dramatically increases during the initial stages of adipogenesis in primary cells and in the

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Supplementary data for this article can be accessed here.

3T3-L1 pre-adipocyte cell line but then decreases to near basal levels upon adipocyte maturity.[19] Moreover, siRNA-mediated knockdown of CD24 in 3T3-L1 pre-adipocytes significantly inhibited the increase in *C/EBPα* and *Pparγ* mRNA expression, and inhibited lipid accumulation compared to control mature adipocytes. However, how CD24 regulates these processes remains unknown.

Thus, we have analyzed the molecular mechanisms regulating adipogenesis by CD24 by using knock-down of CD24 in 3T3-L1 cells as well as primary cells from CD24 knock-out (CD24KO) mice. We found that glucose uptake, the initial step in lipogenesis, was not affected by the knock-down of CD24 in 3T3-L1 cells. However, DNA microarray data suggested CD24 regulates expression of up to 134 genes. Four of these genes were found to be differentially regulated by CD24 in primary cells from inguinal subcutaneous WAT undergoing adipogenesis but only one regulated in cells from epididymal visceral WAT. Therefore, we show here that CD24 alters the regulation of gene expression early during adipogenesis in a depot-specific manner, with no observable impact on glucose uptake.

Results

CD24 regulates lipid accumulation but not glucose uptake in 3T3-L1 adipocytes

We previously found that CD24KO mice have elevated blood glucose levels and hypotrophic adipocytes[21], suggesting that CD24 might regulate lipid accumulation by regulating glucose uptake in adipocytes. Since we also found that CD24 regulated overall lipid accumulation and adipogenesis in 3T3-L1 pre-adipocytes *in vitro*[19], we first analyzed glucose uptake during adipogenesis in 3T3-L1 cells when CD24 was knocked-down. Our previous study validated that CD24 mRNA levels corresponded to protein levels as detected by flow cytometry when regulated endogenously or by siRNA knock-down.[19]

Using a 96-well system to quantify glucose uptake and lipid accumulation concurrently[22], we found that CD24 knock-down by either one of 2 different siRNAs was not effective in all technical replicates. Therefore, to analyze only wells with substantial knockdown of CD24, we compared siCD24 siRNA transfected cells with an expression level of CD24 < 50% of the control scrambled siRNA (Scr siRNA). In addition, only Scr siRNA wells with an expression level of ≥ 70% of the average expression of all Scr siRNA wells were analyzed. A minimum of 2 wells per experiment were included in the analysis. Knock-down of CD24 can only be detected during early phases of adipogenesis, as it returns to baseline expression levels by maturity,[19] therefore, we were only able to verify CD24 knock-down at 6 h and 24 h after IBMX/Dex (I/D) treatment (Figure 1(a-b)). We found, similar to our previous study[19], that analysis of all replicates from the differentiated mature adipocytes showed significantly higher lipid accumulation (as determined by Oil Red O staining) compared to the undifferentiated pre-adipocytes and that transfection of pre-adipocytes with either siCD24 sequence significantly inhibited lipid accumulation compared to the control Scr siRNA in mature adipocytes (Figure 1(c-d)).

However, while uptake of the fluorescent glucose analog 2-NBDG was higher in mature adipocytes compared to undifferentiated pre-adipocytes in 2 independent sets of experiments, there was no effect on glucose uptake in cells with confirmed reduction of CD24 expression at 6 h or 24 h of I/D treatment (Figure 1 (e-f)). In addition, in confluent and mature adipocytes, where CD24 knock-down cannot be confirmed, there was no difference in glucose uptake (Figure 1(e-f)). There was also no significant correlation with CD24 levels and 2-NBDG uptake when all of the data were analyzed irrespective of siRNA treatment (Figure S1). Lastly, knock-down of CD24 did not impact insulin induced glucose uptake (data not shown). Taken together, these results strongly suggest that CD24 does not regulate glucose uptake in 3T3-L1 adipocytes.

CD24 regulates gene expression in 3T3-L1 pre-adipocytes

We then determined if CD24 regulates global gene expression during adipogenesis. By DNA microarray analysis, we found that 134 genes are upregulated in the presence but not in the absence of CD24 (Figure 2 (a)) while downregulation of 41 genes was dependent on CD24 (data not shown). Specifically, DNA microarray analysis identified 134 genes as significantly increased (FDR< 0.05) in control Scr siRNA cells stimulated with I/D for 6 h when compared to untreated (Table 1) but not significantly upregulated in siCD24 cells stimulated with I/D for 6h compared to the untreated.

We selected 11 genes for validation by RT-qPCR. The expression levels of these genes was determined in untreated confluent cells, at initiation of adipogenesis (6 h after I/D treatment) and at maturity (5 d after insulin treatment), and shown as relative to their respective Scr siRNA controls (Figure 2(b-c)). We found that six genes (*Rybp, Fgl2, Fosl1, Itga3, Sh3pxd2a* and *Tiparp*) were not significantly changed when CD24 was knocked-down

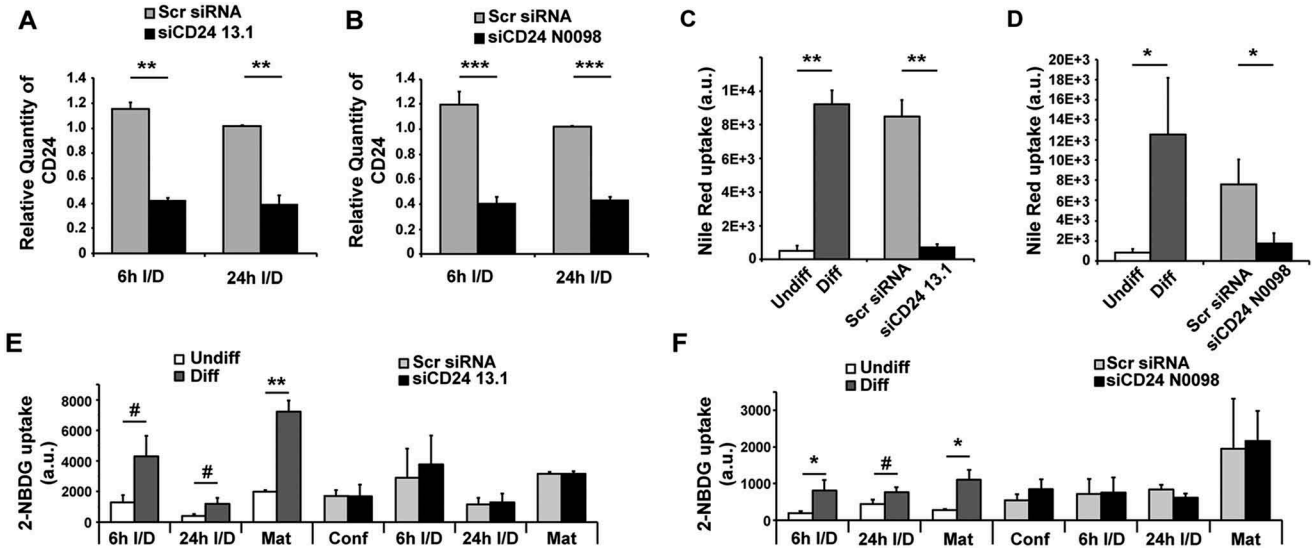

Figure 1. CD24 does not regulate glucose uptake in 3T3-L1 pre-adipocytes during adipogenesis. (a-b) CD24 mRNA levels were quantified by RT-qPCR after cells were transfected with siCD24 siRNA 13.1, siCD24 siRNA N0098 or control Scr siRNA and analyzed at 6 h post IBMX/Dex treatment (6h I/D) or 24h post I/D treatment (24h I/D). For siCD24 13.1, n = 3; for siCD24 N0098, n = 4 for 6h I/D and n = 3 for 24h I/D. Expression normalized to the geometric mean of *RPL-P0* and *ActB* and data shown as $\log_2$ transformed mean ± s.e.m. (c-d) Lipid accumulation detected by Nile Red after cells were transfected with siCD24 13.1, siCD24 N0098 or control Scr siRNA and analyzed at maturity (5 d post insulin). For siCD24 13.1, n = 3, for siCD24 N0098, n = 4. (e-f) Glucose uptake determined by 2-NBDG accumulation in cells not transfected and either left undifferentiated or differentiated as indicated, or were transfected with siCD24 13.1, siCD24 N0098 or control Scr siRNA. 2-NBDG accumulation was analyzed in confluent cells (Conf), 6h I/D, 24 h I/D, or mature cells (Mat), as indicated. n = 3 for all except n = 3–5 for siCD24 N0098 and its corresponding Scr siRNA control. Statistical significance was determined using the student's T-test. Data shown as mean ± s.e.m. $^{\#}p < 0.10$*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. a.u. = arbitrary unit.

when compared to the treated Scr siRNA controls (Figure 2(b)). However, five genes, including both transcript variants of *Ctla2* and *Ccdc85b*, were either significantly different ($p < 0.05$) or approaching significance ($p < 0.1$) at 6 h I/D, at maturity, or at both stages (Figure 2(c)). The expression levels of *Ccdc85b.v2, Ctla2a.v1, Ppm1d,* and *Medag* were significantly lower or approaching significance after 6 h I/D treatment when CD24 was knocked-down. At maturity, *Ccdc85b.v1, Ccdc85b.v2*, and *Rgs2* were significantly lower or approaching significance, while *Ppm1d* was significantly higher, with CD24 knock-down.

CD24 regulates cell autonomous differentiation of the epididymal and inguinal adipocytes in vitro

To confirm the involvement of CD24 in adipogenesis in primary cells from epididymal and inguinal WAT, we first analyzed overall differentiation based on lipid accumulation of SVF isolate from littermate WT and CD24KO male mice. We found that SVF from both WT and CD24KO mice had significant lipid accumulation compared to undifferentiated cells (Figure 3(a-b)). We then calculated the adipogenic potential of the SVF, which we defined as the ratio of Oil Red O absorbance of differentiated cells over undifferentiated cells, to determine the effect of CD24 on differentiation. We found that the adipogenic potential of cells from CD24KO mice was significantly lower in cells from both epididymal and inguinal WAT compared to cells from WT mice (Figure 3(c)). Thus, we can confirm that CD24 regulates overall adipogenesis of primary cells in a cell autonomous manner.

CD24 regulates gene expression in differentiating adipocytes in vitro in a depot specific manner

Previous analysis in 3T3-L1 pre-adipocytes showed that *Pparγ* expression is substantially and significantly decreased, but not abrogated, at maturity when CD24 was knocked down.[19] Therefore, we next analyzed the expression of *Pparγ* during differentiation of primary cells from epididymal or inguinal SVF from WT or CD24KO mice. We found that the *Pparγ* expression was significantly lower at all stages in epididymal and inguinal SVF from CD24KO mice compared to WT (Figure 4(a-b)). Thus, CD24 is also necessary for the full expression of *Pparγ* in primary adipocytes at maturity.

We then investigated the expression patterns of the four genes that we found to be differentially regulated by CD24 in 3T3-L1 pre-adipocytes. We found that in epididymal SVF, the expression of *Medag* was significantly lower in

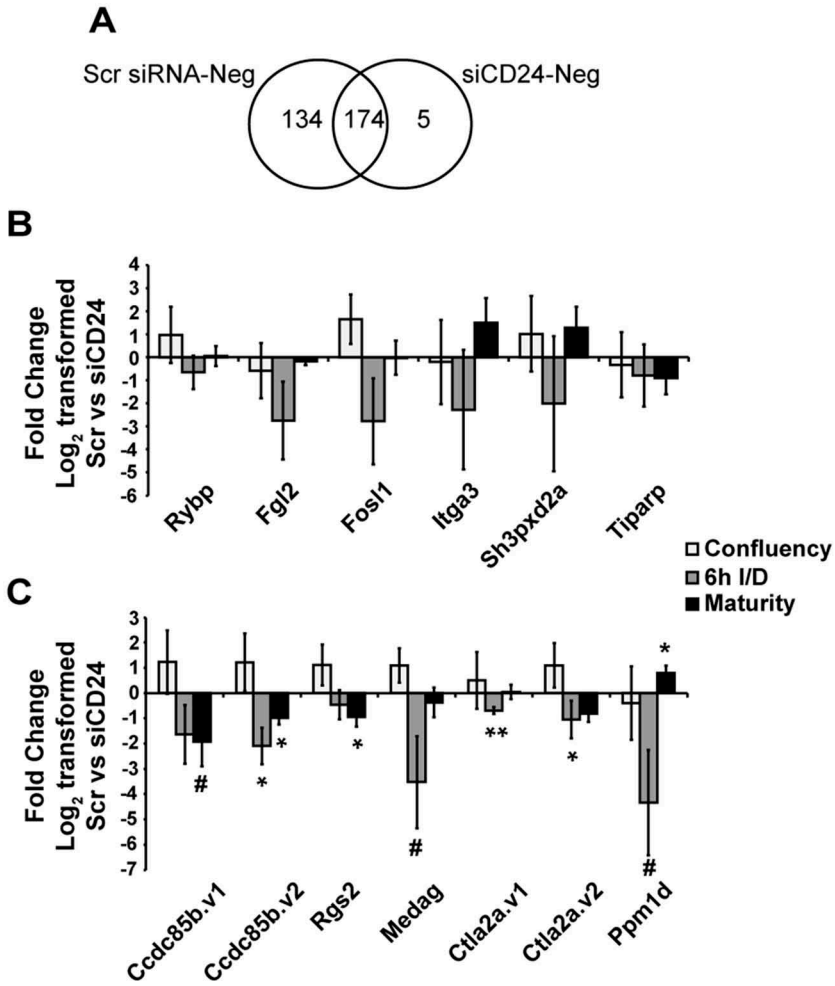

Figure 2. Regulation of gene expression by CD24 in 3T3-L1 pre-adipocytes during adipogenesis. (a) The number of genes upregulated in control Scr siRNA (left circle) or siCD24-transfected (right circle) cells treated with I/D for 6 h compared to vehicle untreated cells (Neg) at FDR< 0.05 as detected by DNA microarray analysis. The left region shows that 134 genes were significantly upregulated in the presence but not absence of CD24. (b) The mRNA expression of 6 genes identified by the microarray were analyzed by RT-qPCR and found not to be significantly altered in the absence of CD24. Data shown as $\log_2$ transformed mean ± s.e.m., n = 4. Internal control genes used for normalization were *Rplp0* and *Gusb*. Relative quantities are shown as ratios with respect to the Scr siRNA controls, which equal 0 on the $\log_2$ scale. (c) The mRNA expression of 7 genes identified by the microarray were analyzed by RT-qPCR and found to be statistically significantly altered in the absence of CD24. Data shown as $\log_2$ transformed mean ± s.e.m., n = 4. Internal control genes used for normalization were *Rplp0* and *Gusb*. Relative quantities are shown as ratios with respect to the respective Scr siRNA controls, which equal 0 on the $\log_2$ scale. The pairwise statistical differences between gene expression in Scr siRNA and siCD24 cells, both treated with I/D for 6 h, was determined using the Student's T-test. $^{\#}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

mature adipocytes from CD24KO mice with no change in expression of the other three genes (Figure 5(a-d)).

In contrast, all four genes were significantly lower at one or more stages of adipogenesis in inguinal SVF from CD24KO mice compared to WT littermates (Figure 6). *Ccdc85b.v2* and *Ppm1d* were significantly lower at 6 h post-confluency in control cells (Control 6 h) and in differentiated cells at maturity (Figure 6(a,d)). *Ctla2a.v1* and *Medag* were found to be significantly lower at every stage of adipogenesis in all differentiating cells at both 6 h and 24 h post-confluency (Figure 6(b-c)).

Therefore, CD24 is necessary for the expression of *Ctla2a.v1, Ccdc85b.v2* and *Ppm1d* in a depot specific manner, whereas *Medag* is regulated by CD24 in both depots.

Discussion

In this study, we found no effect of CD24 on glucose uptake but a regulation of gene expression in early and late stages of adipogenesis by CD24 in manner that was dependent on the depot of origin. In addition, we were able to show for the first time that CD24 regulates adipogenesis of primary cells in a cell autonomous manner. This complements our previous reports of CD24 regulating hyperplasia of adipocytes *in vivo*[21]

Table 1. Expression of 11 genes in response to 6 h ID treatment as determined by DNA microarray.

Gene Name	Gene Symbol	LogFC[a]	Transcript Variants
Regulator of G-protein signaling 2[b]	*Rgs2*	3.438	1
Cytotoxic T-Lymphocyte Associated Antigen 2 Alpha	*Ctla2a*	2.706	2
Fibrinogen-like protein 2	*Fgl2*	2.643	1
Coiled-Coil Domain Containing 85B	*Ccdc85b*	1.704	2
RING1 and YY1 Binding protein	*Rybp*	1.573	1
Mesenteric Estrogen Dependent Adipogenesis	*Medag*	1.495	1
TCDD Inducible Poly(ADP-Ribose) Polymerase	*Tiparp*	1.558	1
Protein Phosphatase, Mg2+/Mn2+ Dependent 1D	*Ppm1d*	1.060	1
SH3 and PX Domains 2A	*Sh3pxd2a*	1.115	1
Integrin Subunit Alpha 3	*Itga3*	0.959	1
FOS Like 1	*Fosl1*	0.884	1

[a]LogFC = Log2 fold change in gene expression level after 6h I/D treatment of Scr control transfected cells compared to untreated cells. FDR < 0.05.
[b]None of these genes had FDR < 0.05 with 6h I/D treatment of siCD24 cells.

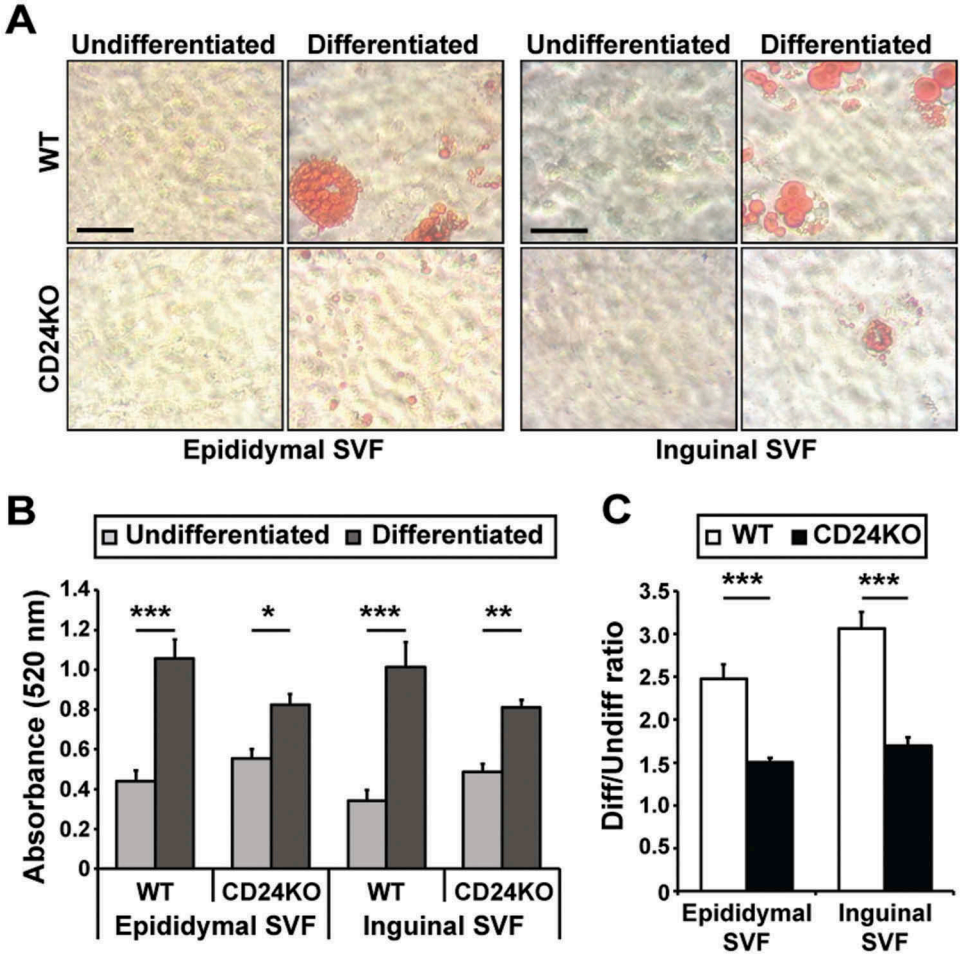

Figure 3. CD24 regulates *in vitro* adipogenesis of stromal vascular fraction (SVF) cells from the inguinal and epididymal depots. (a) Differentiated SVF from inguinal or epididymal WAT obtained from wild type (WT) and CD24KO mice were stained with Oil Red O. Representative images are shown from n = 5–7 animals. Scale bar = 100 μm (b) Oil Red O staining was quantified at 520 nm as indicated. (c) Adipogenic potential was calculated as the ratio of Oil Red O in differentiated (Diff) vs. undifferentiated (Undiff) cells. Data shown as mean ± s.e.m. n = 6 for WT epididymal SVF, n = 5 for WT inguinal SVF, n = 7 for CD24KO. Statistical significance was determined as indicated using Student's T-test. *p < 0.05, **p < 0.01, ***p < 0.001.

and suggests that deficiencies in adipocyte cell size seen *in vivo* are regulated by intrinsic mechanisms. Moreover, although we previously found that fasting glucose levels were elevated in CD24KO mice[21], here we did not find any evidence that CD24 regulates glucose uptake. Thus, together we provide evidence that the increase in circulating glucose levels in CD24KO mice *in vivo* could be consequence of impaired lipogenesis in developing adipocytes rather than directed regulation of blood glucose levels.

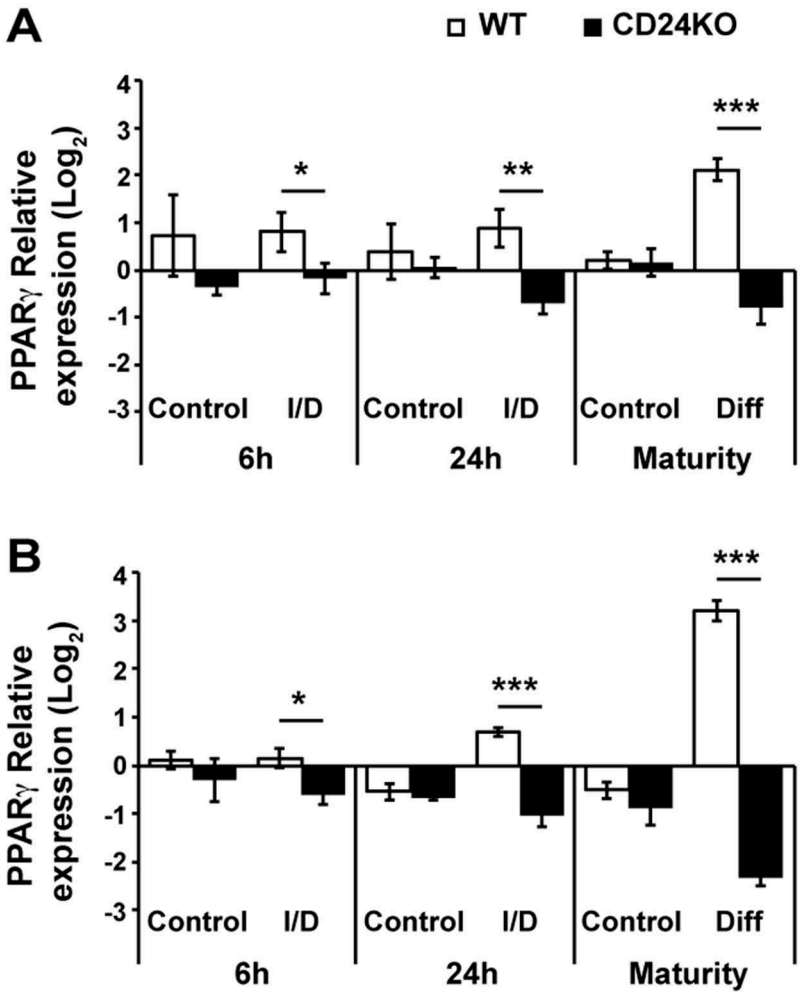

Figure 4. CD24 regulates the expression of *Pparγ* in SVF cells from inguinal white adipose tissue (WAT) and epididymal WAT during adipogenesis *in vitro*. The mRNA expression levels of *Pparγ* in SVF from (a) epididymal SVF and (b) inguinal SVF from wild type (WT) and CD24KO mice were determined. Cells were induced to differentiate as indicated (I/D and maturity) or left undifferentiated for the same amount of time (Control). Gene expression was determined by RT-qPCR followed by normalization to the internal control genes *Rplp0* & *Actb*. Relative quantities are shown with respect to the levels of the pooled calibrator run on each plate. Data shown as $\log_2$ transformed mean ± s.e.m., n = 5. Statistical significance was determined using Student's T-test. *p < 0.05, **p < 0.01, ***p < 0.001.

In support of a cell-intrinsic deficiency, we found that gene expression of multiple genes known to regulate adipogenesis were decreased in 3T3-L1 cells with CD24 knock-down and in inguinal adipocytes from CD24KO mice. These included *Pparγ, Ccdc85b.v2*, and *Medag*. This data confirms our earlier observations that CD24 promotes maximal *Pparγ* expression in mature adipocytes.[19]

Medag is a proinflammatory cytokine that has been shown to promote the expression of *Pparγ*2, *C/EBPα, aP2, C/EBPδ, Plin1, Pref-1* and *Fas* during adipogenesis.-[23] However, it was also observed that glucose uptake is compromized in the absence of *Medag*. We did not observe any alterations in glucose uptake with CD24 knock-down, suggesting that CD24-mediated reduction of *Medag* expression is not sufficient to fully compromize all of the functions of *Medag*. Further studies will be necessary to identify the precise relationship between CD24 and *Medag*.

Ccdc85b, also known as Delta-interacting Protein A, is a transcriptional repressor that inhibits the activity of C/EBPβ and C/EBPδ in pre-adipocytes[24]. Therefore, the decrease in *Ccdc85b* expression during early differentiation and in mature cells with reduced or absent CD24 may cause an increase in C/EBPβ and C/EBPδ activity. It is not clear how this known interaction would result in the observed decrease in lipid accumulation. Future experiments will be required to fully validate any putative causal connection in the context of CD24 expression or activation.

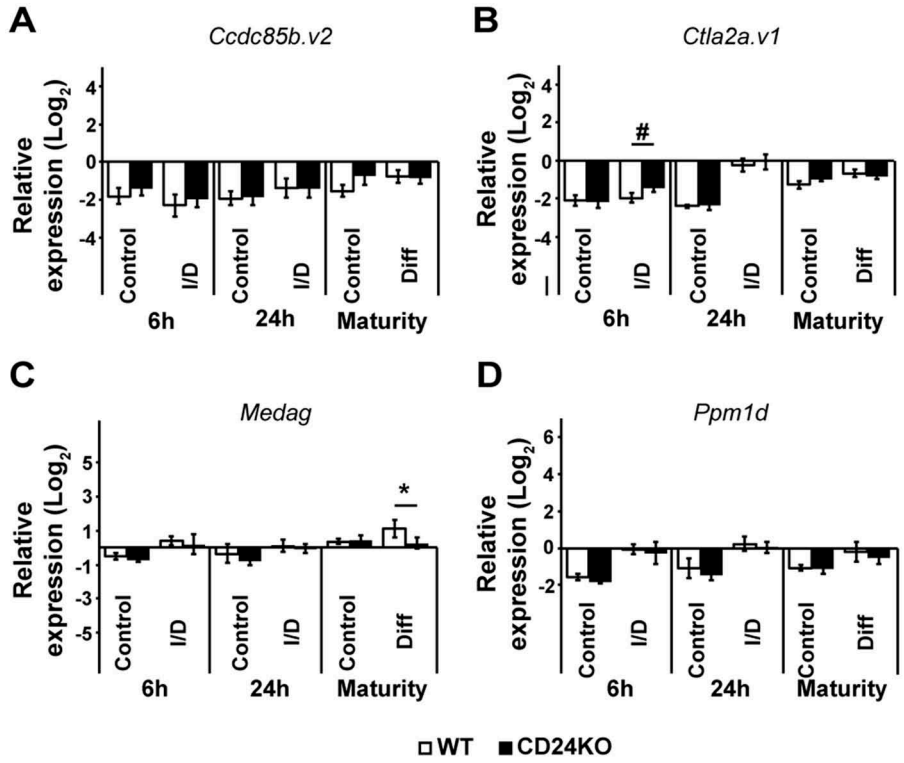

Figure 5. Only *Medag* was significantly regulated by CD24 in SVF cells from epididymal WAT during adipogenesis *in vitro*. The mRNA expression levels of (a) *Ccdc85b.v2*, (b) *Ctla2a.v1*, (c) *Medag*, and (d) *Ppm1d* from epididymal SVF were determined. Cells were induced to differentiate as indicated (I/D and maturity) or left undifferentiated for the same amount of time (Control). Gene expression was determined by RT-qPCR followed by normalization to the internal control genes *Rplp0* & *Actb*. Relative quantities are shown with respect to the levels of the pooled calibrator run on each plate. Data shown as $\log_2$ transformed mean ± s.e.m., n = 5. Statistical significance was determined using Student's T-test. $^{\#}p < 0.10$, $^{*}p < 0.05$.

Reduced CD24 in 3T3-L1 cells and lack of CD24 in inguinal adipocytes resulted in decreased expression of genes that have not yet been reported to contribute to adipogenesis. *Ctla2a* is a cathepsin L-like cysteine protease inhibitor that is expressed in hematopoietic stem cells and can promote apoptosis in T lymphoma cells and cardiac fibroblasts.[25] *Ppm1d* (also known as Wip1) negatively regulates the tumor suppressor p53 to inhibit apoptosis and cell cycle arrest to promote tumorigenesis of cancer cells.[26] It is not clear how regulation of both pro- and anti-apoptotic genes by CD24 at different stages of adipogenesis work together to regulate adipocyte development but suggests that a complicated regulation of inter-connected genes is necessary to allow maximal adipocyte development.

CD24 was necessary for expression of *Ccdc85b.v2, Medag, Ctla2a.v1,* and *Ppm1d* at the pre-adipocyte stage in inguinal SVF and in 3T3-L1 cells, however, the reduction was not statistically significant at the single gene level in all cases. In addition, 6 genes identified by DNA microarray analysis were not validated by the RT-qPCR data. We note that two of the samples that underwent microarray analysis had lower RNA integrity suggesting that the relatively poor correlation between the microarray and the RT-qPCR in from both cell types may be due to RNA degradation (see Materials and Methods). In addition, in order to identify as many potential CD24-regulated genes from the DNA microarray as possible, we compared genes that were differentially expressed in I/D-treated Scr siRNA cells compared to the untreated cells but were not differentially expressed in I/D-treated CD24 siRNA cells when compared to untreated cells. Whereas in the validation RT-qPCR analysis we directly compared the gene expression levels of the genes of interest in treated Scr siRNA vs. treated siCD24 samples; a more robust analysis. Therefore, while the DNA microarray data suggests that regulation of up to 134 genes is dependent on CD24, each gene should be independently verified prior to additional in-depth analysis.

Similar to what we observed previously[19], changes to gene expression was more similar in inguinal SVF and 3T3-L1 cells compared to epididymal SVF. The observation that CD24 is necessary for the expression of these genes well before lipid accumulation is apparent suggests that CD24 may function to control the potential for

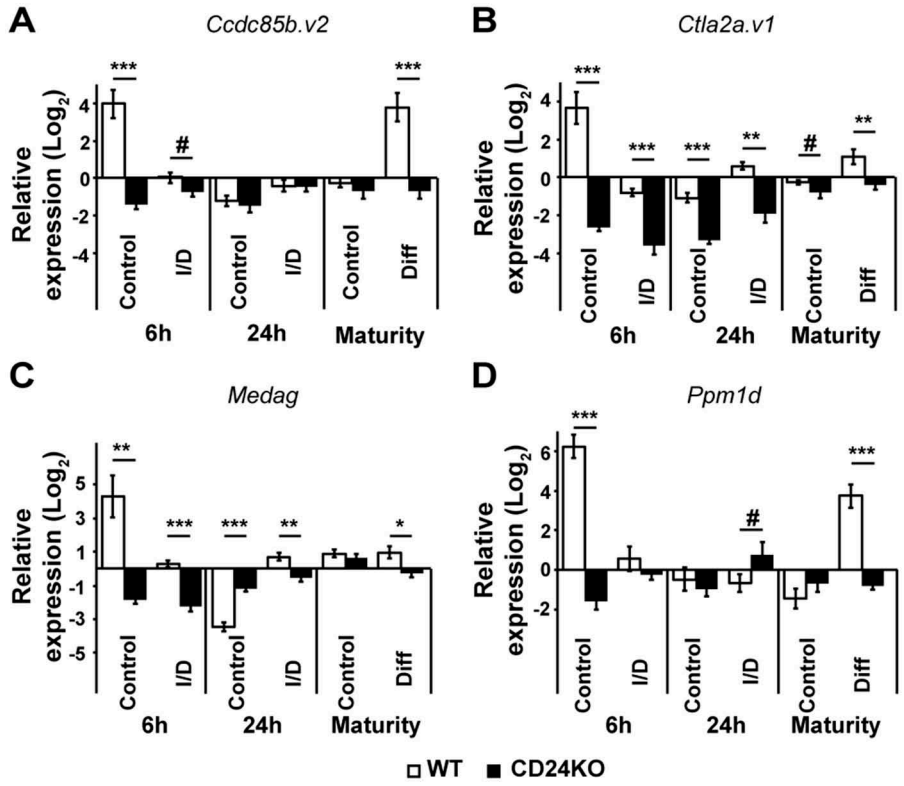

Figure 6. CD24 regulates the expression of *Ccdc85b.v2, Ctla2a.v1, Medag*, and *Ppm1d* in SVF cells from inguinal WAT during adipogenesis *in vitro*. The mRNA expression levels of (a) *Ccdc85b.v2*, (b) *Ctla2a.v1*, (c) *Medag*, and (d) *Ppm1d* from inguinal SVF were determined. Cells were induced to differentiate as indicated (I/D and maturity) or left undifferentiated for the same amount of time (Control). Gene expression was determined by RT-qPCR followed by normalization to the internal control genes *Rplp0* & *Actb*. Relative quantities are shown with respect to the levels of the pooled calibrator run on each plate. Data shown as $\log_2$ transformed mean ± s.e.m., n = 5. Statistical significance was determined using Student's T-test. $^{\#}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differentiation. Moreover, it is likely CD24 modulates adipogenesis by altering the expression of multiple genes in order have a global effect on differentiation as the differences in expression of individual genes was not large. In support of these speculations, the differences in magnitude of each individual gene was highest prior to differentiation and then reduced during the intermediate stages of differentiation followed by an increased difference in mature adipocytes in inguinal cells but not 3T3-L1 cells. The difference in expression in inguinal cells was generally higher than observed in 3T3-L1 cells. Because these cells represent different stages of pre-adipocyte differentiation, and include stem cells in the case of inguinal SVF, this suggests that CD24 may be necessary for both pre-adipocyte commitment as well early stages of differentiation. Lastly, regulation of many different types of genes during early differentiation is the likely cause for the reduction in PPARγ expression and lipid accumulation at maturity. The precise mechanism by which CD24 regulates gene expression, including the signalling pathways and transcription factors regulated by this receptor will have to be elucidated in future studies.

Surprisingly, we did not observe the same alterations to the expression of *Ccdc85b.v2, Medag, Ctla2a.v1,* and *Ppm1d* either in magnitude or significance in epididymal SVF cells, particularly at the pre-adipocyte stage. However, both WAT depots showed a significant reduction in adipogenic potential. It has been reported that there are more stem cells in inguinal SVF compared to epididymal SVF[27] and more recently a difference in regulation of gene expression in stem cells from these depots has been reported[28]. Thus, overall these findings suggest that CD24 could potentially regulate adipogenesis via different mechanisms in the different WAT depots. Future work identifying the contribution of these specific genes as well as additional genes to the regulation of adipogenesis by CD24 may reveal the potential mechanisms that we speculate on here.

In summary, we found that CD24 does not regulate lipid accumulation by regulating glucose uptake in 3T3-L1 pre-adipocytes *in vitro*. Instead, expression

of CD24 is necessary for the upregulation of up to 134 genes in 3T3-L1 pre-adipocytes as determined by DNA microarray analysis. Among the 134 genes, we validated the regulation of four genes by CD24 in 3T3-L1 pre-adipocytes *in vitro*. Further analysis of these genes in SVF from WT and CD24KO mice, showed only *Medag* was dysregulated in the absence of CD24 in the epididymal SVF, while all four genes were dysregulated in inguinal SVF. This is also the first study to show CD24 modulates differentiation of primary cells during adipogenesis *in vitro*. These finding help to elucidate the mechanisms regulating lipid accumulation in adipocytes, which may aid in developing novel treatment strategies for obesity and lipodystophy.

Materials and methods

3T3-L1 cell line and adipogenesis assay

The 3T3-L1 pre-adipocyte cell line was purchased from ATCC and verified to be mycoplasma-free using the MycoAlert Mycoplasma dection kit (Lonza Inc.). 3T3-L1 cells were maintained in high-glucose DMEM supplemented with 10% newborn calf serum (ThermoFisher Scientific) and 1% penicillin/streptomycin (DMEM/NCS; ThermoFisher Scientific). 3T3-L1 cells were grown to confluence for 24 h prior to initiation of adipogenesis.

To initiate adipogenesis, initiation media containing 0.5 mM isobutyl-1-methylxanthine (IBMX; Millipore) and 1 uM dexamethasone (Dex; Millipore) (I/D) in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic (DMEM/FBS) was added to the cells (3T3-L1 cells or primary pre-adipocytes) for 48 h. The media was then replaced with 10 ug/ml insulin (Sigma-Aldrich) in DMEM/FBS for 48 h followed by replacement with DMEM/FBS. The cells remained in this media for an additional 3 d (3T3-L1 cells) or 6 d (primary pre-adipocytes), replacing the media every 2 d, at which time cells could be visually identified as mature lipid-laden adipocytes.

Isolation and culture of primary pre-adipocytes

All animal use was approved by the Institutional Animal Use and Care Committee at Memorial University of Newfoundland (protocol 14–05-SC). The CD24 knock-out (*Cd24*^{atm1Pjln}) mice[29,30] were obtained from Yang Liu, Children's National Medical Center, Washington, DC and backcrossed 5 generations with C57BL/6N (Charles River Laboratories,). Congenic strain background was verified by Charles River Laboratories and heterozygous mice were crossed to obtain male mice homozygous wild type for CD24 or CD24KO littermates. Genotypes were confirmed by PCR and flow cytometry. Mice were weaned onto standard ProlabRMH 3000 rodent chow diet (60% carbohydrate, 26% protein, 14% fat by kcal; Lab Diet) *ad libitum* at 3 weeks of age and continued this diet until 15 weeks of age when the mice were sacrificed. Mice were housed one per cage in micro-isolator cages, on a 12 h: 12 h dark: light cycle, with constant access to water.

The stromal vascular fraction (SVF), which contains pre-adipocytes, among other cell types, but is void of mature lipid filled adipocytes, was isolated from the subcutaneous inguinal WAT and the visceral epididymal WAT of 15-week-old wild type (WT) and CD24KO mice. WAT was minced with a sterile razor blade and digested with 15mg/ml Collagenase type 1 (ThermoFisher Scientific) in Krebs-Ringer-HEPES bicarbonate buffer (pH 7.4) containing 200 nM adenosine and 1% bovine serum albumin (KRH) and incubated for 1 h at 37°C, as previously described.[19,31] Tissue debris was removed by filtering through a 100 µm nylon mesh cell strainer (ThermoFisher Scientific) and SVF collected by centrifuging for 5 minutes at 400 *g*. SVF was washed 3 times in DMEM/FBS, and grown in 24 well plates. Adipogenesis was induced as described above.

siRNA knock down of CD24

CD24 expression was knocked-down (siCD24) as described previously.[19] 3T3-L1 cells were transfected with either 100 nM of CD24 siRNA (MMC.RNAI.N009846.12.1, IDT), or CD24 siRNA (CD24 13.1, IDT) or 100 nM of control scrambled siRNA (Scr siRNA; 51–01-19–08, IDT) using a combination of Jetprime (4 ul for 6-well plate format, 0.2 ul for 96 well plate format) and Interferin (2 ul for 6 well plate fomat, 0.1 ul for 96 well plate format) (PolyPlus Transfection) according to the manufacture's protocol for JetPrime.

Lipid staining

Adipocytes were stained with 0.36% Oil Red O (Millipore) in 60% isopropanol, following the instructions from the Adipogenesis Assay Kit (Millipore), 5 d after insulin addition in 3T3-L1 and 8 d after insulin addition in primary cells. Excess Oil Red O stain was removed by washing the wells three times with 2 ml of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4,

1.8 mM KH2PO4, and pH 7.4). Images were taken after staining at 20X magnification using an Apple iPhone 4 primary camera (5 megapixel with auto-focus) through a 10X magnification eyepiece and images were cropped in ImageJ.[32] The dye was extracted using 500 µl of 100% isopropanol and quantified at 520 nm using the Agilent 8453 Spectrophotometer (Agilent Technologies).

Glucose uptake and lipid accumulation

3T3-L1 pre-adipocytes were grown and differentiated in 96-well plates (Greiner Bio-One) as previously described.[22] The surrounding empty wells were filled with sterile 1X PBS to mitigate evaporation. After reaching 50% confluency, the cells were transfected with CD24 siRNA as described above. To measure glucose uptake, cells were washed with 40 µl of glucose-free serum-free (GS free media) DMEM followed by incubation with 100 µg/ml 2-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) Amino)-2-deoxyglucose (2-NBDG) (Cayman Chemical), a fluorescent glucose analog, in GS free media for 45 minutes at 37°C. Following 2-NBDG incubation, the wells were washed seven times with 1X PBS to remove excess 2-NBDG. Fluorescence was measured immediately using a Synergy Mx fluorescence plate reader (Biotek) at excitation and emission wavelengths of 480 and 550 nm, respectively. After obtaining 2-NBDG fluorescence, mature adipocytes (5 d post insulin treatment), were stained with 10 µg/ml Nile red (Santa Cruz Biotechnology Inc.) and fluorescence was measured on a Synergy Mx fluorescence plate reader at excitation and emission wavelengths of 520 and 580 nm, respectively.

Quantitative reverse transcriptase PCR

All quantitative reverse transcriptase PCR (RT-qPCR) reactions were performed using an Eppendorf Mastercycler Realplex. Primer efficiencies were determined using a 3-fold 4-point dilution series, following MIQE guidelines (Table 2). Normalizer genes were chosen based on stable expression across all treatments as indicated by a 1-way ANOVA analysis.

To analyze knock-down efficiency of CD24 in the 96-well format, cells were lysed and cDNA synthesized following the manufacturer's instructions for the 2-step Cells-to-Ct kit (ThermoFisher Scientific) 6 h or 24 h after IBMX and Dex treatment (I/D). Four microliters of cDNA was used for qPCR with Power SYBR qPCR mastermix (ThermoFisher Scientific). The geometric mean of *Rplp0* and *Actb* was used to normalize the expression level of CD24, taking into consideration the experimentally determined primer efficiencies (Table 2).[33]

For validation of genes from the microarray analysis, qPCR was performed as described previously[19], using Fermentas Maxima SYBR Green qPCR Master Mix (2x) (ThermoFisher Scientific). Concentration of cDNA used is as shown in Table 2 for 3T3-L1 cells and 2 ng used for primary cells. The geometric mean of *Rplp0* and *Actb* was used to normalize the final expression levels of the genes of interest in primary pre-adipocytes, while the geometric mean of *Rplp0* and *Gusb* were used in 3T3-L1 cells, taking into consideration the experimentally determined primer efficiencies (Table 2).[33]

DNA microarray analysis

RNA from 3T3-L1 cells untransfected and untreated cells or cells transfected with Scr siRNA or siCD24 (MMC.RNAI.N009846.12.1) and treated for 6h with I/D was isolated by Smith *et al.*[19] To remove genomic DNA contamination, RNA was treated with Turbo DNA-free (ThermoFisher Scientific) according to the manufacturer's instructions. Isolated RNA (250 ng) was processed and hybridized to the Mouse Gene 2.0 ST GeneChip (Affymetrix, ThermoFisher Scientific) by The Centre for Applied Genomics (TCAG), at The Hospital for Sick Children (Toronto, ON, CA) following the manufacturer's instructions. RNA integrity number was determined using Agilent 2100 Bioanalyzer and found to be greater than 7.9 for all samples except 2 samples which had RNA integrity numbers of 6.2 and 6.9. After consultation with TCAG, all samples underwent cRNA synthesis, labeling, hybridization and scanning as per the manufacturer's instructions.

Data analysis was performed using Bioconductor 2.14 in R 3.1.0.[34,35] The data from 3 independent biological replicates were preprocessed using the Oligo package 1.12.2[36] using the RMA algorithm. Statistically significant changes in gene expression between siRNA or siCD24 transfected cells with 6 h I/D treatment and untreated groups were determined using the empirical Bayes moderated t-test from the Linear Models for Microarray Data (LIMMA)[37] at a false discovery rate (FDR) of 0.05. The data have been deposited in the Gene Expression Omnibus (accession number GSE102403).

Table 2. qPCR primer sequences and efficiencies.

Gene	Sequence	Primer Set Efficiency	cDNA for 3T3-L1 (ng)[a]
Rplp0	F: 5′-TCACTGTGCCAGCTCAGAAC-3′	1.03[a]	n/a[c]
	R: 5′-AATTTCAATGGTGCCTCTGG-3′	1.29[b]	0.4
Gusb	F: 5′-CAGCGGCTGGGCTTTTTAG-3′	0.91[a]	n/a
	R: 5′-CGCTTGCCCTCAACCAAGTT-3′	1.2[b]	10
Actb	F: 5′-CACCCGCGAGCACAGCTTCTT-3′	0.89[a]	2
	R: 5′-TTTGCACATGCCGGAGCCGTT-3′		
Cd24	F: 5′-ACTCAGGCCAGGAAACGTCTCT-3′	1.01[b]	n/a
	R: 5′-AACAGCCAATTCGAGGTGGAC-3′		
Pparγ	F: 5′-TGTTATGGGTGAAACTCTGGG-3′	1[a]	2
	R: 5′-AGAGCTGATTCCGAAGTTGG-3′		
Ctla2a.v1	F: 5′-ACAGGCTGCTCTCCTCAAGT-3′	1[a]	10
	R: 5′-GATGAGCAGGAAGACAGCACTGAA-3′		
Ctla2a.v2	F: 5′-TTGCTCTTCAGAGACCGTGGA-3′	1.04[a]	0.4
	R: 5′-TGCTTTTCTCTGCTCTCACCT G-3′		
Rybp	F: 5′-CCTTTAGGAACAGCGCCGAA-3′	0.9[a]	2
	R: 5′-ATTGATGCGAGGTTTCCTGG-3′		
Rgs2	F: 5′-TGAAGCGGACACTCTTAAAGGA-3′	0.83[a]	0.4
	R: 5′-CTTGCCAGTTTTGGGCTTCC-3′		
Ccdc85b.v1	F: 5′-TCACCTGGGCGAGATCCGT-3′	1.36[a]	0.4
	R: 5′-CTAGCTTCTGCCAACAGCCAC-3′		
Ccdc85b.v2	F: 5′-CTGCCTCTTCCACTGCCTAC-3′	0.98[a]	50
	R: 5′-GGTTCTCCGCCCAGCGCC-3′		
Medag	F: 5′-GTGCCAATCAACCAGTGACA-3′	1.03[a]	2
	R: 5′-GCCCTGATGTCCAGTGTACC-3′		
Ppm1d	F: 5′-GGGCAGATAACACAAGTGCC-3′	1.08[a]	2
	R: 5′-CCCCTGTAGTACTTTGTACCTTGA-3′		
Fgl2	F: 5′-GTCACAGCCGGTTCAACATC-3′	1.05[a]	2
	R: 5′-AGGGGTAACTCTGTAGGCCC-3′		
Fosl1	F: 5′-GCAAGTGGTTCAGCCCAAGA-3′	0.95[a]	2
	R: 5′-CCGTAGTCTCGGTACATGCC-3′		
Itga3	F: 5′-TACCGATGGGCACAGACCTA-3′	1.01[a]	2
	R: 5′-TCCACTTTGGCCCTTGACTC-3′		
Sh3pxd2a	F: 5′-TAGGGAAGCCTTTTCTGCCC-3′	1.05[a]	2
	R: 5′-TTCCCCCACCAACCATCCTA-3′		
Tiparp	F: 5′-AGGACTTCATCCAAGTGCCTG-3′	1.14[a]	2
	R: 5′-AGTTTTCATACTTCTGACTCACCT-3′		

[a] Efficiencies and concentrations using Fermentas Maxima SYBR green 2X mastermix for DNA microarray validation
[b] Efficiencies using Power SYBR qPCR mastermix for quantification of CD24 expression from Cells-to-Ct qPCR analysis.
[c] n/a because equal volumes used.

Statistical analysis

All statistical analyses were performed in R v.3.1.0.[35] Pairwise comparison of parametric data was analyzed by a Student's T-test. When comparing more than 2 treatments, a two-way ANOVA was used followed by a Tukey post hoc test if significant. Differences were considered statistically significant at $p < 0.05$.

Abbreviations

WAT white adipose tissue;
SVF stromal vascular fraction;
ADSC adipose-derived stem cell;
IBMX 3-isobutly-1-methylxanthine;
Dex Dexamethasone;
NBCS New born calf serum;
FBS Fetal bovine serum;
KRH Krebs-ringer-HEPES bicarbonate buffer

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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