

# Programming of adipose tissue miR-483-3p and GDF-3 expression by maternal diet in type 2 diabetes

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Nutrition during early mammalian development permanently influences health of the adult, including increasing the risk of type 2 diabetes and coronary heart disease. However, the molecular mechanisms underlying such programming are poorly defined. Here we demonstrate that programmed changes in miRNA expression link early-life nutrition to long-term health. Specifically, we show that miR-483-3p is upregulated in adipose tissue from low-birth-weight adult humans and prediabetic adult rats exposed to suboptimal nutrition in early life. We demonstrate that manipulation of miR-483-3p levels *in vitro* substantially modulates the capacity of adipocytes to differentiate and store lipids. We show that some of these effects are mediated by translational repression of growth/differentiation factor-3, a target of miR-483-3p. We propose that increased miR-483-3p expression *in vivo*, programmed by early-life nutrition, limits storage of lipids in adipose tissue, causing lipotoxicity and insulin resistance and thus increasing susceptibility to metabolic disease.

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Diet can have important health consequences at any stage of life, but nutrition during fetal and early postnatal life is particularly important as it has major implications for health throughout the life course. The ‘developmental origins of health and disease hypothesis’ proposes that early-life exposure to environmental factors such as suboptimal nutrition has permanent (‘programmed’) effects on the structure and function of key tissues.<sup>1,2</sup> These effects may be beneficial in a situation of continuing nutrient deprivation throughout life. However, they become detrimental in the face of subsequent nutrient excess, making individuals more vulnerable to the effects of an obesogenic diet and increasing the risk of age-associated diseases, especially type 2 diabetes and the metabolic syndrome.

The molecular mechanisms by which nutrition during critical periods of development permanently alters the metabolic phenotype of an organism are poorly defined.<sup>2,3</sup> We and others have addressed this issue using a rat model of maternal protein restriction that exhibits phenotypic similarities to low-birth-weight (LBW) humans, including development of insulin resistance in muscle and adipose tissue and age-dependent loss of glucose tolerance leading to overt diabetes in late adulthood.<sup>4,5</sup> Both adipose tissue and skeletal muscle from nutritionally programmed rats and LBW humans display changes in expression of key insulin signalling proteins before development of insulin resistance.<sup>6,7</sup>

Adipose tissue has been identified as playing a pivotal role in linking early dietary manipulation to long-term metabolic health.<sup>8</sup> Some of the earliest effects and those of greatest magnitude have been demonstrated in adipose tissue.<sup>4,6,7</sup> Adipose tissue is known to play a central role in the regulation of metabolic homeostasis through synthesis and storage of lipids and secretion of adipokines that can modulate metabolism in other tissues. In both rodents and humans, the limited capacity to store lipid in adipose tissue and the consequential ectopic deposition in tissues such as liver and skeletal muscle leads to insulin resistance and metabolic disease.<sup>9</sup> Therefore, the ability to expand adipose tissue and the capacity to store triglycerides is essential in maintaining optimal health.

Many nutritionally programmed changes in protein levels occur in the absence of changes in the level of corresponding mRNAs,<sup>7</sup> indicating a post-transcriptional regulatory mechanism. It is well established that miRNAs can regulate gene expression by influencing mRNA translation without necessarily changing mRNA levels.<sup>10,11</sup> A growing number of miRNAs have been implicated in the regulation of adipose tissue glucose and lipid metabolism<sup>12,13</sup> in adipogenesis<sup>14</sup> and in diabetes.<sup>15,16</sup> However, it remains to be investigated whether miRNAs are susceptible to programming in adipose tissue. Here we address the hypothesis that programmed expression of key miRNAs in adipose tissue mediates effects of early nutrition on adipose cell metabolism and consequently on long-term health. We show that as a consequence of

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**Abbreviations:** GDF3, growth/differentiation factor-3; LP, low protein; RISC, RNA-induced silencing complex; IP, immunoprecipitation; LBW, low birth weight

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upregulation of miR-483-3p in both rats and humans, there is dysregulated expression of one of its targets GDF3 (growth/differentiation factor-3), leading to an inhibition of adipocyte differentiation and maturation. We suggest that this could limit the capacity of adipose tissue to store lipids, leading to ectopic triglyceride deposition, insulin resistance and type 2 diabetes.

## Results

**Adipose tissue mass and adipocyte size are decreased in low-protein rat offspring.** The rat model of maternal protein restriction shows striking phenotypic similarities to LBW humans in terms of both physiology and cellular indices of programming. It is therefore an ideal system to define the molecular pathways linking early nutrition to long-term metabolic health.<sup>7,17</sup> Rat dams were fed a low-protein (LP) diet (8% protein) or a control diet (20% protein) during pregnancy and lactation. Both groups were weaned at 3 weeks onto a normal laboratory chow diet fed *ad libitum*. We studied young adult postpubertal male offspring aged 3 months. At this age, levels of glucose, insulin, triglycerides and cholesterol were not significantly different in LP offspring and controls (Table 1a), although LP offspring become hyperglycaemic in later life.<sup>18</sup> Compared with controls, at 3

months, LP offspring had significantly reduced body weight and reduced body fat, both in absolute terms and as a percentage of body weight (Table 1a). Histology of white adipose tissue shows that this decrease in adiposity is because of the considerably smaller size of adipocytes of LP rats compared with the controls (Figures 1a and b).

**Expression of miRNA-483-3p in adipose tissue is programmed by early-life nutrition.** We analysed miRNA expression in epididymal adipose tissue from LP rat offspring using microarrays at 22 days and 3 months of age (Figure 1c). Only one miRNA was found to be upregulated at both ages miRNA-483-3p (Figures 1c–e), suggesting that this is a sustained programmed event. Interestingly, this miR-483-3p had not been implicated in type 2 diabetes previously and had a number of predicted targets known to influence adipose tissue biology. At 22 days and 3 months, there was a 2.0- and 2.3-fold upregulation of miR-483-3p in LP offspring, respectively, as measured by RT-qPCR (Figures 1d and e). Similar data were obtained by RNase protection assays on different tissue samples from those used to generate the microarray data, and RT-qPCR on kidney, liver and skeletal muscle did not show changes in miR-483-3p expression (data not shown).

To establish the relevance of this observation to human biology, we investigated levels of miR-483-3p in a well-characterised cohort of young adult LBW men (for subject characteristics, see Table 1b). This cohort has been used previously to study the consequences and mechanisms of programming resulting from poor fetal growth.<sup>19,20</sup> We found a twofold increase in miR-483-3p expression in adipose biopsies from LBW men compared with controls (Figure 1f). Similar expression of miR-483-3p in both rats and humans suggests that modulation of miR-483-3p by poor fetal nutrition is a conserved phenomenon.

Within the rat and human genomes the miR-483-3p sequence lies within an intron of the *Igf2* gene. RT-PCR analysis of rat adipose tissue demonstrated expression of IGF2 mRNA from promoters P2 and P3 but not P1 (Figure 1g). There was a small, but significant, increase in expression from the P3 promoter in LP rat offspring (Figure 1g); however, the magnitude of this difference was much smaller than that observed for miR-483-3p. There was no difference in expression from the P2 promoter. This suggests that miR-483 expression can be independently regulated from IGF2.

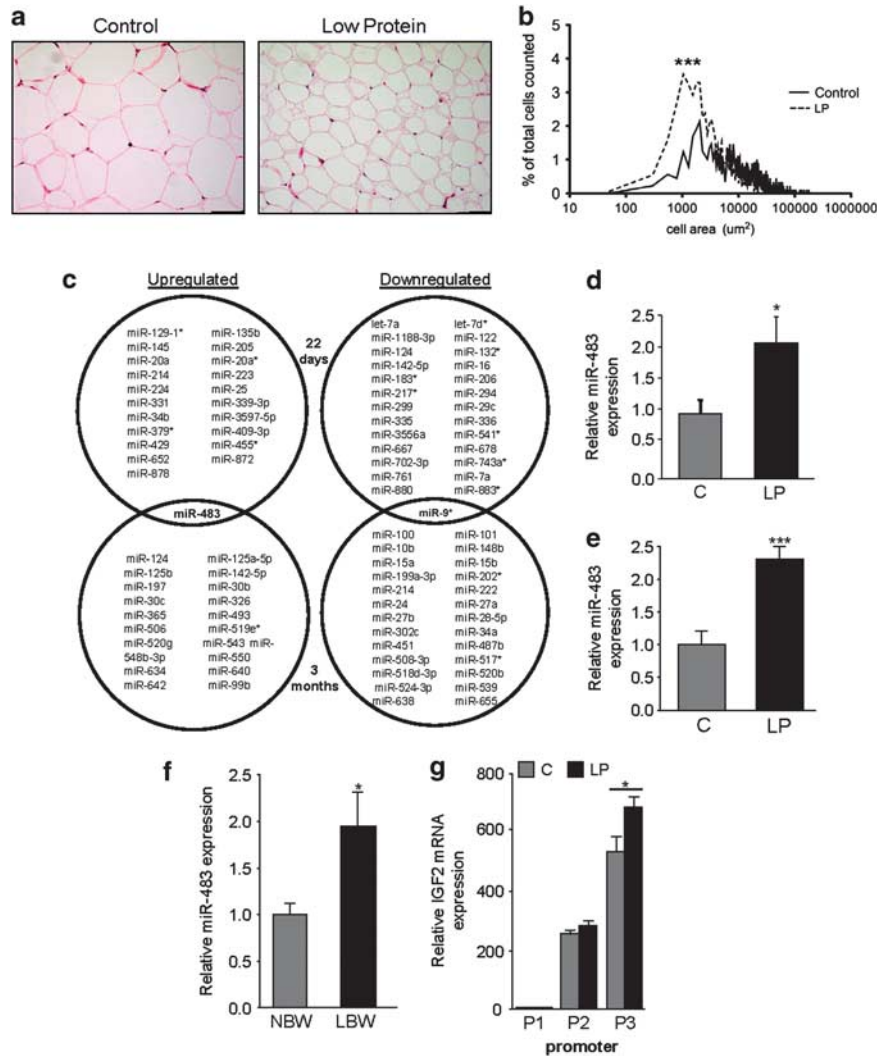
### GDF3 is a potential target of miR-483-3p and is downregulated in LP rat offspring and LBW men.

Examination of online databases (<http://www.targetscan.org/>, <http://www.microrna.org>, <http://www.ebi.ac.uk/enright-srv/microcosm/>) revealed a large number of potential miR-483-3p targets. Candidates were selected for investigation based on potential roles in adipocyte biology, including GDF3, which is expressed at high levels in adult adipose tissue<sup>21,22</sup> and is one of several members of the BMP/TGF- $\beta$  family to have been implicated in regulation of adiposity and energy expenditure as well as cell-fate determination.<sup>23,24</sup> Western blotting of rat adipose tissue revealed a 40% decrease in expression of GDF3 protein (Figure 2a) but no decrease in

**Table 1** Characteristics of (a) rats and (b) humans

	Control		LP	
	Mean	S.E.M.	Mean	S.E.M.
<i>(a) Low-protein and control rat offspring</i>				
Body weight (g)	519	11	416***	8
Epidymal fat weight (g)	9.23	0.31	5.88***	0.22
Epidymal fat (% BW)	1.78	0.06	1.41***	0.05
Intraabdominal fat weight (g)	3.52	0.19	2.47***	0.17
Intraabdominal fat (% BW)	0.68	0.04	0.59	0.04
Retroperitoneal fat weight (g)	11.10	0.58	7.14***	0.40
Retroperitoneal fat (% BW)	2.14	0.11	1.72***	0.10
Glucose (mmol/l)	5.2	0.1	4.8	0.1
Insulin (pmol/l)	96	14	63	12
Total cholesterol (mmol/l)	2.00	0.14	1.97	0.10
Triglycerides (mmol/l)	1.40	0.22	0.99	0.07
Free fatty acids ( $\mu$ mol/l)	1154	98	972	44
HDL (mmol/l)	0.85	0.06	0.86	0.04
LDL (mmol/l)	0.62	0.05	0.85	0.13
<i>(b) Low-birth-weight and control humans</i>				
Age (years)	24.5	0.34	24.1	0.10
Wt (kg)	82.41	3.25	80.66	2.97
Ht (m)	1.86	0.03	1.77*	0.02
BMI (kg/m <sup>2</sup> )	23.93	0.87	25.88	1.14
FFM DXA (kg)	61.93	2.44	58.15	1.53
FFM DXA (%)	20.88	2.15	23.77	2.23
Glucose (mmol/l)	4.66	0.17	5.02	0.15
Insulin (pmol/l)	33.40	6.36	39.33	4.51
C-peptide (pmol/l)	432	64	513	39
TG (mmol/l)	1.01	0.14	1.16	0.13
FFA (mmol/l)	319.00	44	330	30
FPIR (pmol/l)	2147	485	1986	258
M-value clamp (mg/kg FFM/min)	13.24	0.57	14.06	1.07
GOX clamp (mg/kg FFM/min)	5.08	0.32	4.96	0.25
NOGM clamp (mg/kg FFM/min)	8.63	0.39	9.18	1.12

In (a), data are means and S.E.M. Significant differences between control and low-protein (LP) offspring are indicated (\*\*\* $P < 0.001$ ). In (b), data are means and S.E.M. Significant differences between control and low-birth-weight (LBW) subjects are indicated (\* $P < 0.05$ ).

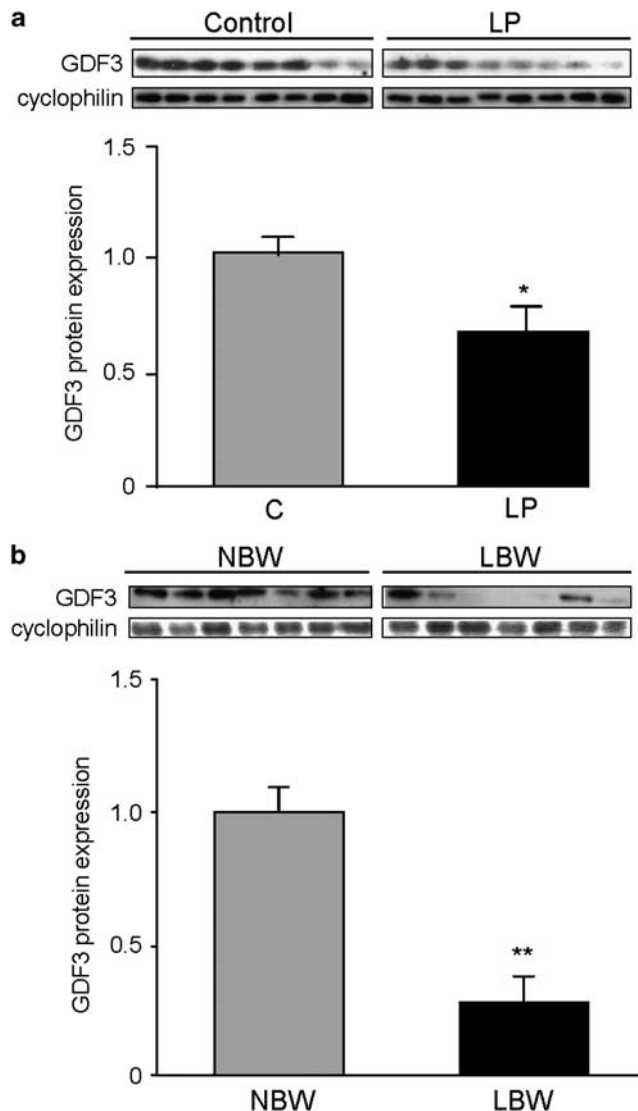


**Figure 1** Adipocyte size is reduced in fat pads of low-protein (LP) rat offspring whereas miR-483-3p expression is increased. (a) Histological images of WAT (bar represents 100  $\mu$ m) showing reduced adipocyte size in LP offspring. (b) Cell sizes quantified as a frequency distribution  $\pm$  95% CI ( $n = 5$  per group,  $***P < 0.001$ ). (c) Venn diagram of up- and down-regulated miRNA expression established by microarrays showing sustained miR-483-3p upregulation through rat maturation. (d) miR-483-3p expression was determined by quantitative PCR in adipose tissue from 3-month-old offspring of LP and control (C) rat dams. miR-483-3p expression is increased twofold in the LP group compared with the control ( $n = 4$  per group). (e) miR-483-3p expression was determined by quantitative PCR in adipose tissue from 3-month-old offspring of LP and control (C) rat dams. miR-483-3p expression is increased 2.3-fold in the LP group compared with the control ( $n = 7$  per group). (f) miR-483-3p expression was determined by quantitative PCR in adipose tissue from young adult men of low birth weight (LBW) or normal birth weight (NBW). The LBW group exhibit a twofold increase in miR-483-3p expression compared with the NBW group ( $n = 10$  per group). (g) Promoter-specific expression of IGF2 mRNA was determined by quantitative PCR in adipose tissue from 3-month-old offspring of LP and control (C) rat dams. IGF2 mRNA expression is under the control of promoters 2 and 3 in adipose tissue. IGF2 mRNA expression dependant on promoter 3 is increased 1.25-fold in the LP group. ( $n = 8$  per group all from different litters).  $*P < 0.05$ ,  $***P < 0.001$

expression of other predicted targets (data not shown). There was no difference between levels of GDF3 mRNA determined by RT-PCR in control and LP adipose tissue (relative levels  $\pm$  S.E.M.  $100 \pm 12$  in controls and  $101 \pm 9$  in LP,  $n = 8$  animals per group), indicating that the effect of maternal diet on GDF3 expression was exerted at a post-transcriptional level. Analysis of protein expression of GDF3 in human adipose tissue revealed a substantial (70%) decrease in GDF3 protein expression in LBW humans compared with controls (Figure 2b). These results demonstrate that the effect of suboptimal early nutrition and growth on both miR-483-3p and GDF3 protein is conserved between humans and rats.

**miR-483-3p directly regulates GDF3 expression by translational repression.** The 3'UTR of GDF3 mRNA contains a predicted miR-483-3p target site corresponding perfectly to nucleotides 2–8 of the mature miRNA. The seed target sequence is conserved between mammals including humans, rat and mouse (Figure 3a) but is not conserved in chicken or *Xenopus* (data not shown). The miR-483-3p seed target site in the human, rat and mouse GDF3 mRNA 3'UTRs is downstream of, but close to, the stop codon (Figure 3a). These localisation features are consistent with previously identified 'hospitable sites' for miRNA interaction.<sup>25</sup>

To examine the relationship between miR-483-3p and GDF3, miR-483-3p mimic was expressed in HEK293 cells



**Figure 2** Adipose tissue expression of GDF3 protein is decreased in low-protein (LP) rat offspring and low-birth-weight (LBW) humans. (a) GDF3 protein expression was determined by western blotting in adipose tissue from 3-month-old offspring of LP and control (C) rat dams. GDF3 protein expression is decreased by 30% in the LP group ( $n = 8$  per group). (b) GDF3 protein expression was determined by western blotting in adipose tissue from young adult men of LBW or normal birth weight (NBW). A 70% decrease in GDF3 protein expression is observed in the LBW group compared with the NBW group ( $n = 7$  per group). \* $P < 0.05$ , \*\* $P < 0.01$

that have low levels of endogenous miR-483-3p. Transfection of miR-483-3p mimic significantly decreased the level of GDF3 endogenous protein (Figure 3b), demonstrating that GDF3 expression is regulated by miR-483-3p.

To establish whether there is a direct interaction between GDF3 and miR-483-3p in the RNA-induced silencing complex (RISC), immunoprecipitation (IP) of the Ago2 protein (a central component of the RISC) was carried out. HepG2 cells that express high endogenous levels of miR-483-3p were used and the Ago2 IP was carried out in the presence or absence of miR-483-3p antagonist. The total RNA from the Ago2 IP was isolated and RT-qPCR carried out to quantify the

levels of miR-483-3p and GDF3 mRNA present. Importantly, it was demonstrated that both miR-483-3p and GDF3 mRNA were associated with Ago2 (Figure 3c). Whereas miR-483-3p association with Ago2 was not changed significantly in the presence of an antagonist, GDF3 mRNA association with Ago2 was completely abrogated by a miR-483-3p antagonist (Figure 3c). These results demonstrate that both miR-483-3p and GDF3 mRNA associate with the RISC and that the binding of GDF3 mRNA is dependent on miR-483-3p.

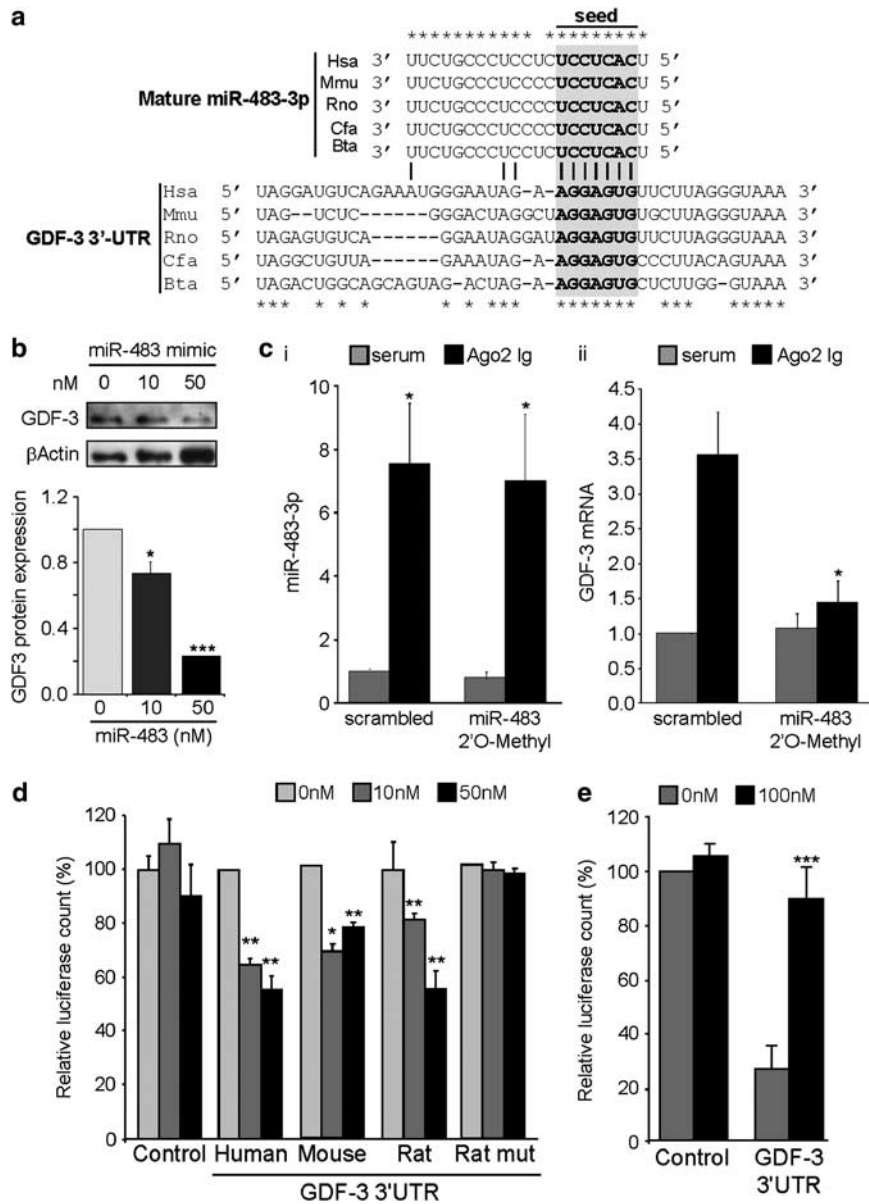
To confirm that the predicted seed sequence for miR-483-3p is mediating the repressive effect on GDF3 translation, the 3'UTR of human, rat and mouse GDF3 cDNAs encompassing the predicted site were cloned into a luciferase-based reporter plasmid. The constructs were transfected into HEK293 cells, together with increasing concentrations of a miR-483-3p mimic. Luciferase activity was decreased significantly in the presence of the mimic in all species (Figure 3d). The specificity of this effect was shown by introducing point mutations within the miR-483-3p target seed sequence of the rat 3'UTR that made it insensitive to the presence of miR-483-3p (Figure 3d). In addition, transfection of an antagonist to miR-483-3p in HepG2 cells relieved the repression of the luciferase reporter under the control of the 3'UTR from the GDF3 transcript (Figure 3e).

Taken together, these data confirm the presence of a functional and direct miR-483-3p target site in the 3'UTRs of the mouse, rat and human GDF3 mRNA.

**miR-483-3p regulates adipocyte differentiation and lipid accumulation.** Having established a relationship between miR-483-3p and GDF3, it was then important to investigate the expression patterns of these two components during the adipocyte differentiation process using the murine 3T3-L1 cell line. Consistent with our observations that miR-483-3p directly regulates GDF3, we demonstrated that during adipocyte differentiation, the expression of miR-483-3p was inversely correlated with that of GDF3. The expression of miR-483-3p decreased gradually to 50% of its original levels by day 9 of differentiation (Figure 4ai). In contrast, GDF3 mRNA and protein expression increased significantly during this period (Figure 4aii and iii). GDF3 protein was virtually undetectable in undifferentiated cells (day 0) and after 4 days of differentiation, but was highly expressed after 7 and 9 days of differentiation, suggesting that it does not play a role in early stages of the differentiation process. It is important to note that although GDF3 mRNA increased approximately twofold during differentiation, the protein expression showed an eightfold increase (Figure 4aiii), consistent with post-transcriptional regulation.

To define the role of miR-483-3p in adipocyte differentiation and lipid accumulation, the effect of modulating miR-483-3p was investigated in the 3T3-L1 cell line. Expression of miR-483-3p mimic substantially inhibited adipocyte differentiation as defined by expression of FABP4 and PPAR $\gamma$ 2 mRNAs. The expression of these mRNAs was dramatically reduced throughout the differentiation process in the presence of the miR-483-3p mimic (90% decrease for both markers at day 9) and, importantly, significantly increased in the presence of miR-483-3p antagonist (two- and three-fold increase at day 9 for PPAR $\gamma$ 2 and FABP4, respectively; Figure 4b). Immunohistochemical



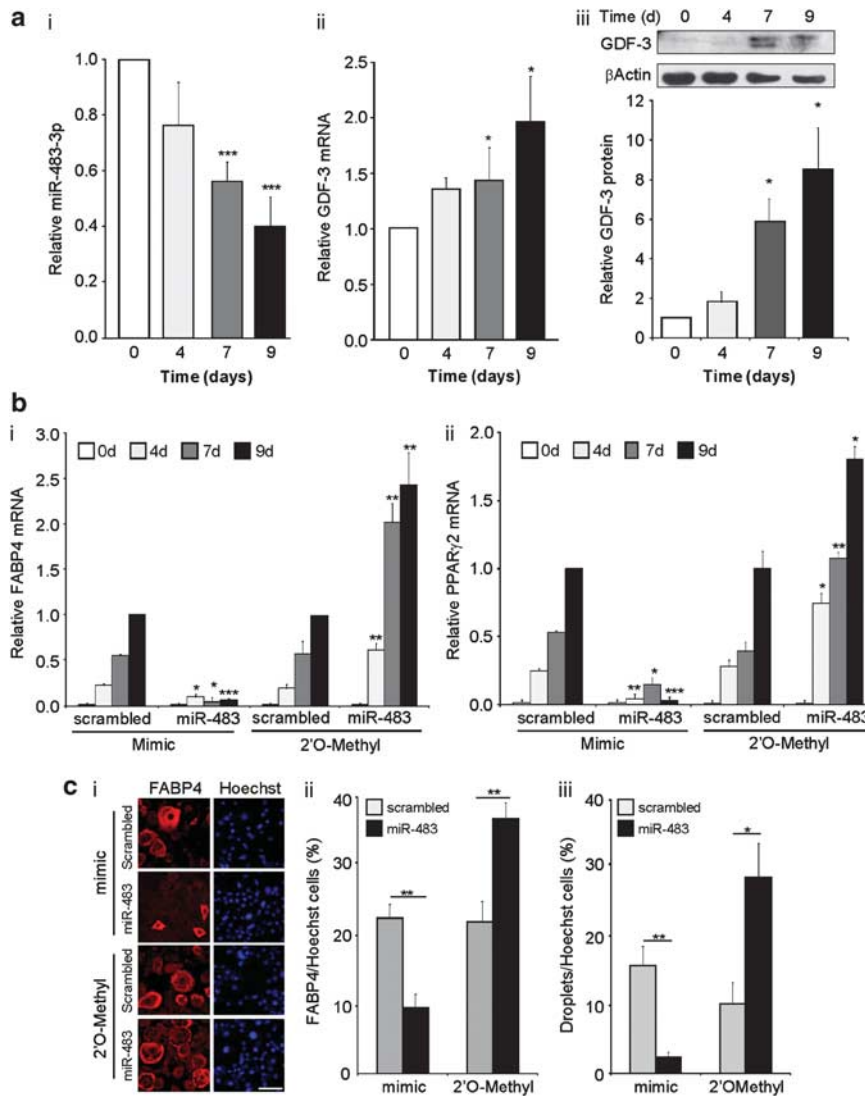


**Figure 3** GDF3 protein expression is controlled by miR-483-3p. (a) Sequence alignment of miR-483-3p and human, mouse, dog, bovine and rat GDF3 mRNA 3' UTR. The miR-483-3p seed and target sequences are highlighted. (b) HEK293 cells were transfected with a mimic of miR-483-3p. GDF3 protein expression was evaluated by western immunoblotting. (c) HepG2 cells expressing high levels of miR-483-3p were transfected with a miR-483-3p 2'-O-Methyl antagonist. Ago2 protein immunoprecipitation was performed and total RNA was collected. RT-qPCR was performed to assay the levels of miR-483-3p and GDF3 mRNA levels present in the immunoprecipitated RISC complex. (d) HEK293 cells were co-transfected with luciferase reporter constructs containing a portion of the 3'UTR of GDF3 mRNA spanning the miR-483 seed target region (human, mouse, rat wild-type or rat with mutated seed sequence (Rat mut)), together with 0, 10 or 50 nM miR-483-3p mimic. (e) HepG2 cells were co-transfected with luciferase reporter constructs containing a portion of the 3'UTR of rat GDF3 mRNA spanning the miR-483 seed target region, together with 0 or 100 nM miR-483-3p 2'-O-Methyl miR antagonist. Data are mean of 4–8 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus mimic or antagonist

studies (Figure 4ci) showed that transfection of miR-483-3p mimic decreased the number of FABP4-positive cells by 50% while decreasing the number of lipid droplet-containing cells by 80% (Figure 4c) on day 9 of differentiation. Sequestration of miR-483-3p by the use of the antagonist had the opposite effect and increased the amount of FABP4-positive and lipid droplet-containing cells twofold (Figure 4c).

Given the effects of miR-483-3p on GDF3 expression and that GDF3 is one of several members of the BMP family to

have been implicated in the regulation of adipocyte size and expandability,<sup>21,23</sup> the influence of GDF3 on differentiation and lipid accumulation was examined *in vitro*. Specific targeting of GDF3 mRNA using siRNA resulted in decreased induction of FABP4 and PPAR $\gamma$  mRNAs on days 7 and 9 of differentiation (Figure 5a), with no significant effect at earlier time points, consistent with the absence of GDF3 at earlier stages of differentiation (Figure 4a). Knockdown of GDF3 also decreased the number of FABP4-positive cells and



**Figure 4** miR-483-3p modulates adipocyte differentiation. (a) Levels of miR-483-3p (i) and GDF3 mRNA (ii) were measured by RT-qPCR from differentiating 3T3-L1 pre-adipocytes whereas protein level of GDF3 was assayed by western immunoblotting (iii). (b) 3T3-L1 cells were transfected with miR-483-3p mimic or 2'-O-Methyl miR-483-3p antagonist and expression of FABP4 (i) and PPAR $\gamma$ 2 (ii) was determined by quantitative PCR after 0, 4, 7 and 9 days (d) of differentiation. (c) 3T3-L1 cells were transfected with miR-483-3p mimic or 2'-O-Methyl miR-483-3p antagonist. On day 9 of differentiation, cells were stained with Hoechst reagent (blue) or labelled anti-FABP4 antibody (red) (i). For each experimental condition, panels show the same field of view for both staining reagents. Histograms represent percentage of cells marked with FABP4 (ii) or percentage of cells containing lipid droplets (iii) relative to stained nuclei. Data are mean of 3–5 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus scrambled control

reduced the number of lipid-containing cells by 70% on day 9 of differentiation (Figure 5b).

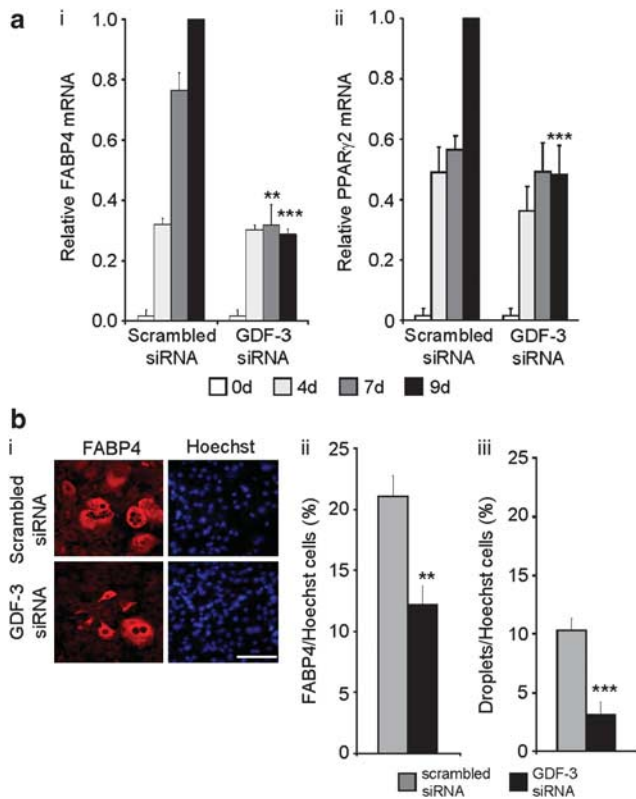
Taken together, these data strongly suggest that miR-483-3p represses the expression of GDF3 that is required for lipid accumulation at the late stages of differentiation.

## Discussion

It is well established that suboptimal early growth and nutrition is associated with increased risk of diseases such as type 2 diabetes in later life. However, the molecular mechanisms by which events during critical periods of development permanently alter the physiology and metabolism of an organism, following multiple rounds of cell division, are poorly defined.

We, and others, have demonstrated previously that programmed changes in DNA methylation and histone modifications affecting transcription of 'gatekeeper genes', such as transcription factors, contribute to this cellular memory.<sup>26,27</sup> However, there is strong evidence to suggest that post-transcriptional mechanisms also play a critical role in nutritional programming. The data presented herein demonstrate that sustained programmed changes in miRNA permanently influence gene expression and tissue physiology, providing a novel post-transcriptional mechanism by which early environmental factors control long-term metabolic health.

Programming the expression of key miRNAs (each with multiple targets) provides a mechanism by which coordinated



**Figure 5** GDF3 modulates adipocyte differentiation. (a) 3T3-L1 cells were transfected with siRNA targeting GDF3, and expression of FABP4 (i) and PPAR $\gamma$ 2 (ii) was determined by quantitative PCR after 0, 4, 7 and 9 days (d) of differentiation. (b) 3T3-L1 cells were transfected with siRNA targeting GDF3. On day 9 of differentiation, cells were stained with Hoechst reagent (blue) or labelled anti-FABP4 antibody (red) (i). Histograms represent percentage of cells marked with FABP4 (ii) or percentage of cells containing lipid droplets (iii) relative to stained nuclei. Data are mean of 3–5 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus scrambled control

regulation of gene networks can be achieved through central nodes of regulation. Our observation that the changes in miRNA expression in both rats and humans occur before the development of the metabolic phenotype suggests that they are causative and do not arise as a consequence of metabolic dysfunction. This also raises the possibility that they could be used as markers of disease risk if such changes were identified in a clinically accessible tissue. Our study identified a number of miRNAs that were differentially expressed in adult rat offspring as a result of alterations in maternal diet, and we focused on miR-483-3p as it was the only maintained programmed change that occurred at 22 days and 3 months and it has not previously been implicated in the development of type 2 diabetes or regulation of metabolic health. Our data suggest that programming of miR-483-3p is fundamentally important as levels of miR-483-3p were increased in adipose tissue from LP rat offspring and in LBW humans, showing that programming of miR-483-3p was therefore conserved between species. Modulation of miR-483-3p levels *in vitro* revealed that this miRNA regulates early and late stages of differentiation as well as the capacity of adipocytes to store lipids. Therefore, dysregulation of miR-483-3p expression could have a substantial impact on whole body physiology by

directly inhibiting adipose tissue expandability and lipid storage, which would in turn affect other tissues by promoting ectopic triglyceride storage and lipotoxicity. Consistent with this hypothesis, development of diabetes in the LP rat model is not associated with increased adiposity, and although older animals exhibit increased hepatic lipid accumulation,<sup>28</sup> they do not become obese.<sup>4</sup> It is also well documented that LBW humans are at increased risk of nonalcoholic fatty liver disease.<sup>29</sup> We therefore suggest that upregulation of miR-483-3p in adipose tissue of rat LP offspring and LBW men contributes to the metabolic imbalance leading to diabetes by limiting their capacity for triacylglyceride storage in adipocytes and therefore enhancing ectopic lipid accumulation in other tissues including muscle and liver. By limiting adipose tissue expandability, increased expression of miR-483-3p would make such programmed individuals very vulnerable to the detrimental effects of an obesogenic diet.

The mechanisms that link programmed changes in expression of miRNAs, each with multiple potential targets, to metabolic phenotype and disease susceptibility are likely to be exceedingly complex. Our bioinformatics and experimental approach identified GDF3 as a target that mediates the effects of miR-483-3p on adipose tissue. We showed that levels of GDF3 protein were reduced in adipose tissue from both LP rat offspring and LBW humans, and that manipulation of GDF3 expression influenced the later stages of differentiation and lipid accumulation in 3T3-L1 cells. However, GDF3 protein was almost undetectable in undifferentiated 3T3-L1 cells and during the first few days of differentiation, and therefore the effects of miR-483-3p on the early stages of differentiation are likely to be mediated through other as yet unidentified targets.

Consistent with our observations that the reduction in GDF3 in LP offspring is accompanied by a decrease in adipose cell size, it has been shown previously that compared with wild-type animals, GDF3-deficient mice have an ~40% decrease in adipocyte size when fed a normal chow diet and exhibit an obesity-resistant phenotype when fed a high-fat diet.<sup>21,22</sup> In adipocytes, GDF3 appears to signal through an ALK7/Cripto receptor complex, and ALK7-deficient mice also demonstrated decreased expansion of fat cell mass and smaller cell size when fed a high-fat diet.<sup>21</sup> Moreover, mice overexpressing GDF3 exhibited increased weight gain on a high-fat diet compared with control animals and this was accompanied by increased adipocyte size.<sup>30</sup> GDF3 is expressed in both mature adipocytes and stromal/vascular cells,<sup>22</sup> and therefore it might act in either a paracrine or autocrine manner.

The mechanisms through which the programmed increase in miR-483-3p arises are currently unknown. miR-483 is an intragenic miRNA, located in intron 2 of the *Igf2* gene,<sup>31</sup> which is paternally imprinted.<sup>32</sup> Altered methylation of the locus has been linked to programmed changes in expression of IGF2 in an ovine model of periconceptual undernutrition,<sup>33</sup> however, this study in sheep did not assess the levels of miR-483. Although we observed increased expression of IGF2 mRNA from one of the *Igf2* gene promoters (P3) in adipose tissue of LP offspring, the magnitude of the change in IGF2 mRNA was much less than that of miR-483-3p, and expression of IGF2 transcripts from the other main promoter were not different. Independent regulation of IGF2 and miR-483 in adult tissues would be expected given that IGF2 transcript levels in adult

rats are very low.<sup>34</sup> Expression of mouse miR-483\* was reported to be downregulated, whereas IGF2 was upregulated in the liver of offspring of dams fed a high-fat diet during pregnancy and lactation,<sup>33</sup> and a recent study showed that expression of miR-483-3p can be induced independently of IGF2 by  $\beta$ -catenin and promoter-region methylation *in vitro*.<sup>35</sup> Indeed, it is well established that miRNA expression can be regulated independently from the host gene either through the use of an alternative promoter, differential processing or through modulation of transcript stability.<sup>36</sup>

In conclusion, we propose that by modulating adipose tissue expandability and thus the ability of the individual to store lipids within adipose tissue, programmed changes in miRNAs contribute to the relationship between early-life nutrition and long-term health. These findings not only contribute to our understanding of fundamental biological processes, but also have important implications for improving human health as programmed miRNAs represent potential markers of disease risk as well as providing targets of rational therapeutic intervention strategies.

#### Materials and Methods

**Animals.** The LP rat model has been described in detail previously.<sup>37</sup> Virgin female Wistar rats weighing 240–260 g were housed individually and maintained at 22°C on a 12 h light–dark cycle. They were mated and day 1 of gestation was taken as the day on which vaginal plugs were expelled. The pregnant dams were then randomised into two dietary groups, control and LP, fed *ad libitum* and weighed daily. The control group received a 200 g protein/kg diet and the LP group received an isoenergetic 80 g protein/kg diet, as described.<sup>38</sup> Dams were maintained on their respective experimental diets throughout pregnancy and lactation. At 48 h after delivery, litters were reduced at random to 8 pups with an equal gender ratio where possible. At 21 days of age, all pups were weaned onto standard chow (LAD1 diet, Special Diets Services, Witham, UK) and fed *ad libitum*. The animals were then maintained on this diet throughout the study. At 22 days and 3 months of age, a single male offspring from each litter was fasted overnight, and the next day blood was taken for glucose measurements (OneTouch Ultra, LifeScan Inc., Milpitas, CA, USA) and serum was obtained for determination of fasting insulin (Ultrasensitive mouse insulin EIA kit, Mercodia, Uppsala, Sweden) and lipid analysis (Mouse Biochemistry Laboratory, Department of Clinical Biochemistry, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK) (Table 1). The rats were then killed by CO<sub>2</sub> asphyxiation. Total body weights were recorded and individual fat depots were collected and weighed and then either snap-frozen in liquid nitrogen and stored for miRNA, mRNA and western analysis or fixed for histological analysis. In all cases, 'n' refers to the different numbers of litters represented in the analysis. All studies were approved by the Local Ethics Committee and were conducted according to the Home Office Animals (Scientific Procedures, UK) Act 1986.

**Human subjects.** A total of 20 human male volunteers born in Copenhagen County between 1979 and 1980 after 39–41 weeks of gestation were recruited from The Danish National Birth Registry according to birth weight. All subjects participated in a recently published study.<sup>39</sup> In all, 10 men had birth weights below the 10th percentile for gestational age (LBW) and 10 men had birth weights in the upper normal range (50th–90th percentile) (Control). The LBW subjects had a slightly lower adult height but otherwise there were no significant differences between groups in clamp insulin action (80 mU/m<sup>2</sup>/min insulin infusion, 3 h), *in vivo* insulin secretion or DXA scan body composition (Table 1b). None of the subjects had a family history of diabetes (parents or grandparents) or received medication known to affect glucose or lipid metabolism. Subjects with BMI > 30 kg/m<sup>2</sup> were excluded. Lifestyle factors such as tobacco use, exercise and diet were not different between groups, as assessed by an interview at inclusion. Subcutaneous fat biopsies were obtained from the abdomen in the fasting basal state before the clamp during local anaesthesia using a modified Bergstrom needle. Biopsies were blotted free of blood, frozen immediately in liquid nitrogen and stored for later analysis at –80°C. The study protocol was reviewed and approved by

the regional ethical committee and procedures were performed according to the principles of the Helsinki declaration. Written informed consent was obtained from all participants.

**Microarray analysis.** Microarray slides were prepared in the laboratory of Dr. Tim Gant based on the microRNA database found on miRBase v9.6 (<http://www.mirbase.org/>). FlashTag 3DNA array detection kit (Genisphere, Hatfield, PA, USA) was used to label 1  $\mu$ g of total RNA (Oyster-550) and 10 ng of MirExplore microRNA universal reference (Oyster-650, Miltenyi Biotec, Cologne, Germany). Arrays were scanned using an Axon4200 scanner and data were compiled using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA). Analyses were carried out on control and LP adipose tissue samples (four per group). These samples were obtained from a separate cohort of animals to those used in validation studies.

**Immunoblotting.** Adipose tissue (250 mg) was powdered on dry ice and homogenized in 300  $\mu$ l lysis buffer (50 mM HEPES (pH 8), 150 mM NaCl, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 30 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM EDTA with protease inhibitors (set III, Calbiochem Novabiochem Biosciences, Cambridge, UK)). Total protein concentration of lysates was determined by a copper/bicinchoninic assay (Sigma Aldrich, Poole, UK). Total protein (20  $\mu$ g) from each sample (each animal and each litter represented by one sample; n = 8 Controls, n = 8 LP) was subjected to SDS-PAGE along with protein markers (Fermentas, York, UK). Separated proteins were transferred onto PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). Protein blots were incubated in blocking buffer before incubation with primary antibody according to the manufacturers' protocols. Primary antibodies used were against rat GDF3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or human GDF3 (Abcam, Cambridge, UK). This was followed by incubation with horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and detection by chemiluminescence (SuperSignal West Pico, Pierce, UK). Autoradiographs were analysed by spot-densitometry (AlphaEase, AlphaInnotech, San Leandro, CA, USA). Signals from 20 and 10  $\mu$ g of a pooled sample confirmed linearity of exposure.

**qRT-PCR analysis.** RNA from rat and human adipose tissue was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) whereas RNA from cultured cells was isolated using the Trizol reagent protocol (Invitrogen, Paisley, UK). The extracted RNA was purified by phenol–chloroform phase extraction and re-precipitated using isopropanol. Isolated RNA was DNase treated and further purified using RNAeasy columns (Qiagen, Crawley, UK). For mRNA expression analysis, cDNA synthesis was primed using random hexamers (Promega, Southampton, UK). Specific primers for quantification were designed using the Universal Probe Library Assay Design Center (Roche, Welwyn Garden City, UK) and target specificity confirmed with Primer-blast (NCBI). qRT-PCR was performed using the Sybr green master mix on the 7500 Fast Real Time PCR system (Applied Biosystems, Warrington, UK). Expression was normalised to that of the housekeeping genes *cyclophilin* and  $\beta$ -*actin*, which did not differ between experimental groups. RT-qPCR of microRNAs was performed using TaqMan MicroRNA assay kits for hsa-miR-483-3p, rno miR-483 and snU6 rRNA (Applied Biosystems) according to the manufacturer's protocol.

#### Primer list:

IGF2 (total) sense sequence: 5'-CGCTTCAGTTTGCTGTTCCG-3'  
antisense sequence: 5'-GCAGCACTCTCCACGATG-3'  
IGF2 (P1) sense sequence: 5'-TTGCCACACAGTTTTCCCATTT-3'  
antisense sequence: 5'-TCCCAAATCAGACCCCTTGTC-3'  
IGF2 (P2) sense sequence: 5'-CAAGTGGATTAATTATACGCTTTCTG-3'  
antisense sequence: 5'-AGAGGCGGGTAGGCTCAC-3'  
IGF2 (P3) sense sequence: 5'-TGAGAACCTCCAGCCTTTTCTGTC-3'  
antisense sequence: 5'-CTGAAGTTATAAGGATAAGGAAFFCCGCT-3'  
GDF3 sense sequence: 5'-ACACACAGAAACCTTCCCAAG-3'  
antisense sequence: 5'-TTCTTTGACGGCAGACAAGTT-3'  
FABP4 sense sequence: 5'-CCCTCCTGTGCTGACGCTT-3'  
antisense sequence: 5'-TGGCCATGCCTGCCACTTTCC-3'  
PPAR $\gamma$ 2 sense sequence: 5'-GCCTTCCTGCTGATGCACTGCC-3'  
antisense sequence: 5'-TGCGAGTGGTCTCCATCACGG-3'  
Cyclophilin sense sequence: 5'-CCCATCTGCTGCAATA-3'  
antisense sequence: 5'-TTTGCAATCCTGCTAGACTTGA-3'  
 $\beta$ -Actin sense sequence: 5'-CGACAACGGCTCCGGCATGT-3'  
antisense sequence: 5'-TCACACCTGGTCCCTAGGGC-3'



**Luciferase reporter constructs and assay.** Luciferase reporter constructs<sup>40</sup> were generated by cloning the 3'UTR portion of human, mouse and rat GDF3 mRNA containing the seed target sequence of miR-483-3p that is highly conserved across species (Figure 3a). PCR of HEK293 cDNA library using specific primers yielded 181 of 199 bp of human 3'UTR, PCR of mouse embryonic stem cell cDNA yielded the first 200 of 900 bp of mouse 3'UTR whereas PCR on rat white adipose tissue cDNA yielded 200 of 724 bp of rat 3'UTR. These PCR products were then subcloned downstream of the luciferase gene contained in the pGL3-Basic commercial vector (Promega). Mutation of the seed target sequence of the rat GDF3 3'UTR from AGGAGTG to AGGATAG was performed using the Quickchange site-directed mutagenesis kit (Agilent Technologies, Edinburgh, UK).

HEK293 cells were used for miR-483-3p expression studies, and HepG2 cells for miR-483-3p knockdown studies, because of their low and high levels of endogenous miR-483-3p, respectively. Cells were transfected with 100 ng of reporter construct, 50 ng of p- $\beta$ -Gal-control (Clontech, Basinstoke, UK) used as transfection control and increasing concentrations of miR-483-3p mimic (0, 10 and 50 nM in HEK293) or miR-483-3p 2'-O-Methyl antagonist (0 and 100 nM in HepG2) using the transfection reagent RNAi Max (Invitrogen). Cells were harvested and Luciferase assay was performed using LAR-I substrate (Promega). A  $\beta$ -Galactosidase Tropic luminescence (Applied Biosystems) assay was performed as a transfection efficiency control. Luminescence was detected using the GloMax system (Promega).

**Ago IPs.** HepG2 were transfected with 100 nM of miR-483-3p 2'-O-Methyl antagonist or a scrambled 2'-O-Methyl RNA sequence. The cells were then harvested in PBS and then put in the presence of 1% formaldehyde for 1 h to establish protein-RNA reversible crosslinking. Cells were lysed and sonicated. The protein lysate was immunoprecipitated using a specific Ago2 mouse monoclonal antibody (Abcam) and bound by A/G agarose beads (Santa Cruz Biotechnology). Beads bound to the antibody were resuspended in water after precipitation and heated at 75 °C for 45 min to disrupt RNA-protein bounds. RNA was then extracted and purified using Trizol protocol.

**3T3-L1 culture, differentiation and treatment.** Monolayer cultures of 3T3-L1 mouse preadipocytes were grown to confluence in DMEM supplemented with 10% newborn bovine serum. Cells were then treated with DMEM supplemented with 10% fetal bovine serum (FBS), IBMX (0.5 mM), dexamethasone (1  $\mu$ M) and insulin (5  $\mu$ g/ml) for 48 h. Medium was then removed and replaced with DMEM containing 10% FBS and insulin (5  $\mu$ g/ml). After 48 h, medium was removed and replaced with 10% FBS DMEM and changed subsequently every 48 h.

Cells were transfected with 20 nM miR-483-3p mimic, 50 nM miR-483-3p 2'-O-Methyl antagonist or 20 nM siRNA targeting GDF3 using RNAi max as a transfectant (Invitrogen) 24 h before first treatment and again 24 h before second treatment. Cells were harvested at days 0, 4, 7 and 9 of the differentiation protocol.

**Immunohistochemistry of 3T3-L1 cells.** After 9 days of differentiation 3T3-L1 cells were fixed on coverslips using 4% paraformaldehyde and permeabilised using 0.1% Triton X-100. FABP4 protein was stained using a specific monoclonal antibody (Cell Signaling Technology/NEB, Hitchin, UK) and a secondary anti-mouse antibody conjugated with Alexa-488 fluorophore. The nuclei were stained with Hoechst reagent.

**Adipocyte cell size and number analysis.** Adipose tissue was obtained from epididymal fat pads of 3-month-old male Control and LP rat offspring ( $n = 5$  per group). The samples were fixed in paraformaldehyde, embedded in paraffin, cut into 5  $\mu$ m sections and stained with haematoxylin and eosin. Histological images (bar represents 100  $\mu$ m) were digitally captured and individual cross-sectional areas of adipocytes measured and quantified using Cell:D imaging analysis software (Olympus, Southend-on-Sea, UK). Cell sizes were then presented as frequency distribution histogram ( $\mu$ m<sup>2</sup>) and cell number per field at  $\times 4$  magnification. Data are then presented as means (95% CI); \*\*\* $P < 0.001$  by two-tailed  $t$ -test.

**Statistics.** Data were analysed by unpaired Student's  $t$ -tests using Prism 5 (GraphPad, La Jolla, CA, USA). For all the parameters studied in rodents, 'n' refers to the number of litters, from each of which one animal was taken. For all data sets,  $P < 0.05$  was considered statistically significant. Human miR-483-3p expression data were not normally distributed, and were log transformed before analysis.

## Conflict of Interest

The authors declare no conflict of interest.

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