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“MicroRNA Expression Profiling in human Subcutaneous Adipose Tissue”

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Introduction
1. Obesity

1.1. Physiological basis of obesity

Obesity is a global epidemic and contributes to the increasing burden of type 2-diabetes, cardiovascular disease, stroke, some types of cancer, and premature death worldwide. Our organism is generally more efficient in starvation response since this condition is a higher threat to survival; however, in many experimental animals and several humans, increase in energy storage promotes long-term and short-term adaptive responses that lead to resistance to obesity. These responses, as loss of appetite or increase energy expenditure, use the same effector mechanisms that act in response to starvation in opposite direction: unfortunately, in current environment, obesity-suppressing stimuli are insufficiently robust to prevent obesity and its complications.

Obesity is caused by perturbations of the balance between food intake and energy expenditure, which is regulated by a complex physiological system that requires the integration of several peripheral signals and central coordination in the brain. The hypothalamus functions as a central regulator in this system. It receives information about energy balance through neuronal and hormonal signals to several tissue nuclei within it - particularly the ventro-medial, paraventricular and arcuate nuclei - and to the lateral hypothalamic area (Xu, B. et al, 2003) (Fig.1). The arcuate nucleus has an essential role in this system; it contains two sets of neurons, one produces agouti-related protein (AGRP) and neuropeptide Y (NPY) and the other produces pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART). The first type are orexigenic, promoting food intake and reducing energy expenditure, and the second type produce the opposite anorexigenic effect (Barsh et al., 2002). Peripheral endocrine signals exert their effects through this system over both long- and short-term time-frames. Insulin, secreted from beta cells in the pancreas in response to elevated blood concentrations of glucose, has a role in the Central Nervous System as a
signal of the level of adiposity over a moderate- to long-term period and has an anorexigenic effect through stimulation of POMC/CART neurons and inhibition of AGRP/NPY neurons (Air, E. L. et al, 2002). The anorexigenic hormone leptin, an adipocyte-derived cytokine, seems to be the principal adiposity indicator and signal of the state of nutrition, as its plasma levels are highly correlated to adipocyte number and fat content (Friedman et al., 1998). However, leptin is involved in the long-term regulation of adiposity, and other peptides are responsible for the short-term regulation of appetite. One of these, the orexigenic peptide ghrelin, is secreted primarily from the stomach and duodenum, and shows a rise in serum levels before eating and a decrease after eating (Kohno et al., 2005). Another mediator, peptide YY3–36 (PYY3–36), is secreted from the distal gastrointestinal tract on ingestion of food, with concentrations peaking within approximately 1 hour. It binds to presynaptic Y2 receptors in the NPY neurons that have putative inhibitory effects, which might lead to decreased food intake. Satiety is mediated by the response to other factors, such as gut distension and the release of the peptide cholecystokinin (CCK) (Spiegelman et al., 2001).

The central arcuate nucleus processes these different inputs and exerts its effects by signalling to various downstream effector neurons. These include the orexigenic melanin-concentrating hormone (MCH) neurons and orexin or hypocretin neurons in the lateral hypothalamus (Spiegelman et al., 2001), the thyrotrophin-releasing hormone (TRH) neurons that are involved in regulating the hypothalamic–pituitary–thyroid axis (Flier et al., 2000) and the α-aminobutyric acid (GABA)-releasing interneurons in the paraventricular nucleus (PVN), which modulate orexigenic or anorexigenic effector neurons.

Further inputs to this system include the dopamine, serotonin and endocannabinoid signalling systems (Fig. 1).
Physiological regulation of energy balance.
The neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons, and the pro-opiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) neurons in the arcuate nucleus of the hypothalamus have key roles in the regulation of energy balance. Activation of the NPY/AGRP neurons has an orexigenic effect, promoting food intake, whereas the POMC/CART neurons have the opposite anorexigenic effect.

Figure 1 | Physiological regulation of energy balance.
The neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons, and the pro-opiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) neurons in the arcuate nucleus of the hypothalamus have key roles in the regulation of energy balance. Activation of the NPY/AGRP neurons has an orexigenic effect, promoting food intake, whereas the POMC/CART neurons have the opposite anorexigenic effect.
This weight-regulatory system is a powerful protection against weight loss; however, the same cannot be said for weight gain (Schwartz et al., 2003).

Signals from fat to brain regulate many processes including appetite and energy expenditure. With increasing adiposity, the consequent rise in leptin has a limited effect on reducing food intake and averting obesity (Considine et al., 1996).

The anti-obesity role of leptin is limited by cellular resistance to this signal, which might have developed in response to evolutionary pressure to promote fat storage and so protect against starvation (Spiegelman et al., 2001).

Analysis of physiological pathways has highlighted possible candidate genes that might underlie the genetic basis of obesity. In general, genetic studies have contributed significantly to understanding the physiology of weight regulation, through both the use of animal models and the investigation of the genetics of rare and common human forms of obesity.

1.2. Genetic of human obesity

Obesity is an important cause of morbidity and mortality in developed countries, and is also becoming increasingly prevalent in the developing world. Although environmental factors are important, there is considerable evidence that genetic predisposition also has a significant role in its pathogenesis. The identification of genes that are involved in monogenic, syndromic and polygenic forms of obesity has greatly increased our knowledge of the mechanisms that underlie this condition (Bell et al., 2005).

It is well established that mutations in genes that encode proteins with likely roles in appetite regulation are responsible for Mendelian disorders in which obesity is the most obvious phenotype.

Monogenic forms of obesity are represented from mutations in the gene that encode leptin (Zhang et al., 1994), the leptin receptor (LEPR) (Tartaglia et al., 1995),
carboxypeptidaseE (responsible for processing prohormone intermediates, such as proinsulin) (Naggert et al., 1995), Pro-opiomelanocortin (POMC) and melanocortin-4 receptor (MC4R) (Huszar et al., 1997).

A key role of leptin in some monogenic forms of obesity was further supported by the striking effect of leptin replacement in an extremely obese child with congenital leptin deficiency. In this 9-year-old child, the daily subcutaneous injection of recombinant human leptin for a year led to a complete reversal of obesity, with sustained fat-mass loss (Farooqi et al., 1999). However, only a handful of families with extreme forms of obesity in early infancy have mutations in these genes.

However, individuals with monogenic forms of obesity represent only a small fraction of the obese population and many other candidate genes have been evaluated to contribute to polygenic forms of obesity (Loos and Bouchard, 2003). Many studies have demonstrated that polymorphisms located in genes and candidate genetic loci concur to determine an individual’s susceptibility to weight gain (Clement, 2006).

With the increasing scale of the obesity problem and the body of evidence outlined above for a strong genetic contribution to development of the disease, many groups have started to study the genetics of polygenic obesity to better understand the pathogenesis of the disease and highlight possible pharmacological targets. Two approaches that are commonly used across the whole field of complex human genetics have been used to identify the underlying genes: candidate-gene and whole-genome approaches. However, many other genetic alterations contribute to development of obesity phenotype.

At least 20 rare syndromes that are caused by discrete genetic defects or chromosomal abnormalities, both autosomal and X-linked, are characterized by obesity.
Most of these obesity syndromes are distinguished by the presence of mental retardation, severe hyperphagia, and/or other signs of hypothalamic dysfunction, indicating an origin at the level of the CNS (Delrue et al., 2004).

The most frequent of these syndromes (1 in 25,000 births) is Prader–Willi syndrome (PWS), an autosomal-dominant disorder that is characterized by obesity, hyperphagia, diminished foetal activity, muscular hypotonia, mental retardation, short stature and hypogonadotropic hypogonadism caused by deletion at the chromosomal region 15q11.2–q12 (Jiang et al., 1998), sometimes associated with the deletion or disruption of the single minded homologue 1 (SIM1) gene, in humans (Holder et al., 2000). Pseudohypoparathyroidism type 1A (PHP1A) syndrome is due to a maternally transmitted mutation in GNAS1, which encodes the α-subunit of the Gs PROTEIN.

Food-intake abnormalities in patients with this syndrome might be due to the expression of the resulting variant Gs protein in the hypothalamic circuitry that controls energy balance, which involves many G-protein coupled receptors (Spiegel et al., 2004).

The candidates discussed here and listed in Table 1 are only a small selection of those published, and a more complete list can be found at the Obesity Gene Map database (Obesity Gene Map database: http://obesitygene.pbrc.edu). As would be expected, owing to both experimental and statistical variation, there have been studies that have reported no association for all of these genes; however, from the available functional data, they are all plausible candidates for genes that are involved in common obesity.

These genes are involved in the regulation of energy metabolism, appetite control or autocrine–paracrine signalling by adipocytes.

It is clear from the descriptions above that the molecular causes that underlie the aetiology of syndromic obesity are more complex than for monogenic cases, and further studies are required to elucidate their genetic basis.
Table 1: A selective list of genes that are associated with obesity phenotypes

The list includes genes for which markers have been shown to be associated with obesity-related phenotypes in multiple published studies. A more complete list can be found at the Obesity Gene Map Database (see Online links box).

*Official gene symbols and names are from the HUGO nomenclature committee web site (http://www.gene.ucl.ac.uk/nomenclature). ‡Chromosomal band location is as listed in the NCBI database. ||Short-hand descriptions of associated phenotypes that are statistically significant at p < 0.05. BMI, body mass index; WHR, weight-hip ratio.

| Gene* | Gene name* | Location† | Phenotypes measured‡, || |
|-------|-------------|-----------|------------------|-------------|
| ACDC  | Adipocyte, C1Q and collagen domain containing, adiponectin | 3q27 | BMI, waist circumference, BMI | |
| ADRA2A| Adrenergic receptor α-2A | 10q24–q26 | Skinfold ratio, abdominal fat, Skinfold ratio | |
| ADRA2B| Adrenergic receptor α-2B | 2p13–q13 | Basal metabolic rate, weight-gain | |
| ADRB1 | Adrenergic receptor β-1 | 10q24–q26 | Weight, fat mass, BMI | |
| ADRB2 | Adrenergic receptor β-2 surface | 5q31–q32 | WHR, obesity, BMI, subcutaneous fat accumulation, obesity, Adipocyte lipolysis | |
| ADRB3 | Adrenergic receptor β-3 | 8p12–p11.2 | WHR, BMI, weight-gain capacity, earlier onset | |
| LEP  | Leptin (obesity homologue, mouse) | 7q31.3 | Obesity, BMI | |
| LEPR | Leptin receptor | 1p31 | BMI, fat mass, overweight, fat mass, overweight, fat mass | |
| NR3C1 | Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) | 5q31 | Obesity, overweight | |
| PPARG | Peroxisome proliferative activated receptor, γ | 3p25 | BMI, weight, fat mass, BMI, overweight, fat mass | |
| UCP1 | Uncoupling protein 1 (mitochondrial, proton carrier) | 4q28–q31 | Weight, BMI, WHR | |
| UCP2 | Uncoupling protein 2 (mitochondrial, proton carrier) | 11q13 | Obesity, BMI, obesity, skinfold thickness | |
| UCP3 | Uncoupling protein 3 (mitochondrial, proton carrier) | 11q13 | Caloric intake, fat intake, fat mass, WHR, BMI, Skinfold thickness, BMI | |
2. Gene expression in adipose tissue

Obesity is highly heritable and arises from the interactions of multiple genes, environmental factors, and behaviour. Epidemiologic, and then molecular, studies confirmed this pathogenesis: from 30 to 80% of weight variation are related to genetic factors and moreover, that more than 50% of all gene expression traits in adipose tissue are strongly correlated with clinical traits related to obesity (Emilsson et al., 2008).

A different gene expression emerged in adipose tissues of obese subjects respect to those of lean individuals also in dependence of sex, diet, exercise and hormonal factors. By microarray technology a change of expression at transcriptome level in adipose tissue has been previously evaluated (Gomez-Ambrosi et al., 2003; Viguerie et al., 2005). A general down-regulation of lipolysis-inducing genes and up-regulation of mitogen-activated protein kinases (MAPK) have been observed together with a general increase of the expression of inflammatory cytokines and immune system-related genes (Gomez-Ambrosi et al, 2003). The metabolic and endocrine functions of adipose tissue from various depots differ in a way that may explain the association of visceral but not subcutaneous fat with obesity-related cardiovascular and metabolic problems (Arner, 1998).

Regarding the metabolic function of fat, visceral adipose tissue is more sensitive to the stimulation of lipolysis by cathecolamines, whereas subcutaneous fat is more sensitive to the antilipolytic effects of insulin. Concerning endocrine function, visceral and subcutaneous adipocytes have different capacities to produce hormones and enzymes. Depot-related variation in mRNA expression has been shown for several genes, including leptin, TNF-α, angiotensinogen, PAI-1 (Arner, 1998), and recently, carboxypeptidase E and thrombospondin-1 (Ramis et al., 2002). The mechanisms responsible for depot differences in adipose function are unknown. It is possible that fat cells in various regions have different origins and, because of this, express different
genes (Fig. 2). Recent indirect evidence supports this idea, because newly formed adipocytes in human subcutaneous and visceral fat were shown to maintain the phenotypic site differences of mature adipocytes (Van Harmelen et al., 2002).

3. MicroRNA

3.1. Biogenesis

Gene regulatory factors that control the expression of genomic information come in a variety of flavors like microRNAs representing the most numerous gene regulatory factors in multicellular genomes (Hobert et al., 2008). These molecules are non-protein-coding small RNAs (microRNAs) are approximately 21–23 nucleotides in length, and act by negatively regulating gene expression at the post-transcriptional level (Lagos-Quintana et al. 2001, Lau et al. 2001, Bartel 2004). Mature miRNAs are derived from two major processing events, driven by sequential cleavages by the RNAse-III enzymes Drosha and Dicer (He & Hannon 2004 and Kim 2005). Briefly, miRNAs are transcribed by RNA polymerase II, producing primary-miRNAs (pri-miRNAs) (Lee et al. 2002, 2004).

Pri-miRNAs, often several kilobases long, are polyadenylated and capped, similar to the production of mRNAs from protein encoding genes (Cai et al. 2004, Lee et al. 2004). These pri-miRNAs are then subjected to processing by the microprocessor complex, composed of Drosha and its associated binding partner, Pasha (also known as DGCR8 in mammals), which results in the excision of a 65–75 nucleotide stem-loop precursor called a pre-miRNA (Lee et al. 2003, Landthaler et al. 2004).

These pre-miRNAs are then recognized and transported from the nucleus to the cytoplasm via the Ran-GTP dependent nuclear transmembrane protein, Exportin5, where they are than subjected to a second cleavage step by Dicer (Yi et al. 2003, Lund et al. 2004).
Figure 2 | Schematic overview indicating genes that may be implicated in the mediation of the pathological consequences of obesity or involved in the genetic susceptibility to obesity.
Processing by Dicer results in the production of a small double-stranded miRNA duplex containing 2-nucleotide-long 3’ overhangs (Bernstein et al. 2001). These double-stranded products are thought to be quickly unwound by an as yet unidentified helicase, and a single mature strand can be asymmetrically incorporated into the RNA-induced silencing complex (RISC) (Khvorova et al. 2003, Schwarz et al. 2003) where they can then act by translational repression (by a cleavage-incompetent RISC) or mRNA degradation (by a cleavage-competent, Slicer-containing RISC) (Tang, 2005). MicroRNA can have two mechanisms for post-transcriptional gene silencing and they act by mediating translational repression or degradation of the mRNA targets. In animals, most miRNAs are thought to function by translational repression, whereas in plants mRNA degradation is thought to be the primary mechanism by which they exhibit their silencing effects. Translational repression is thought to occur when the miRNA imperfectly pairs to the target mRNA, although the precise mechanism has yet to be elucidated (Fig. 3).

The degree of repression is often correlated with the number of miRNA binding sites, most of which are thought to occur in the 3’ untranslated regions (UTRs) of the targets, but which can potentially bind to complementary sequences in the open reading frame (ORF) or 5’ UTR (Doench & Sharp 2004). In contrast, mRNA degradation is thought to occur by siRNA-acting miRNAs. siRNA-acting miRNAs form perfect/near perfect interactions with their target mRNAs, which results in the cleavage of the mRNA known as RNA interference (Fig. 3) (Rhoades et al. 2002). This perfect/near perfect association presumably triggers the action of Slicer, which in mammals is putatively thought to be the Argonaute2 (Ago2) endonuclease acting either alone or in addition to other Argonaute or as yet unknown proteins within the RISC complex (Liu et al. 2004b, Meister et al. 2004, Okamura et al. 2004).
MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human let-7a-1 miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.) RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22-nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left). However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally found in the 3' untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.
Slicer functions by cleaving the target mRNA between the 10th and the 11th nucleotide from the 5' end of the miRNA (Tuschl et al. 1999, Liu et al. 2004a). Thus far, only a handful of mammalian miRNAs have been shown to act in a siRNA-like manner. One example is miR-196, which directs the cleavage of the HOXB8 transcript (Mansfield et al. 2004, Yekta et al. 2004), and it will be interesting to determine if there are others that can act by this mechanism. Intriguingly, knockout of Ago2 in mice is lethal as it is required for development, suggesting that a cleavage-competent RISC is essential in mammals (Liu et al. 2004b). This finding, along with the evolutionary conservation of a cleavage-competent RISC suggests that there may be more siRNA-acting microRNAs.

3.2. Function

MicroRNAs have been originally describe in Caenorhabditis elegans (miRNA family, lin-4) and they have been viewed this RNA as an oddity in worm genetics (Lee et al. 1993). Seven years passed before the discovery of another C. elegans miRNA, let-7, which also was found to regulate developmental timing in these animals (Reinhart et al. 2000). This time, however, the discovery was particularly intriguing because let-7 is highly conserved among bilaterally symmetrical animals (Pasquinelli et al. 2000).

Accumulating studies have confirmed that about 1,000 of these RNAs are encoded in animal genomes, and the most recent computational studies estimate that up 30% of human genes may be regulated by microRNA 12-13 and also distinct miRNAs in humans, together potentially regulating a large portion of protein-encoding genes (and possibly non-protein-encoding genes as well) (Berezikov et al. 2005). Now, it knows that miRNAs play an important role in translation regulation in plants, Drosophila and mammals (Table 2).
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Potential Gene</th>
<th>Biological processes involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7</td>
<td>Lin-41</td>
<td>Limb development</td>
<td>Schulman et al. 2005</td>
</tr>
<tr>
<td>miR-196</td>
<td>Hoxb8</td>
<td>Limb development</td>
<td>Hornstein et al. 2005</td>
</tr>
<tr>
<td>miR-143</td>
<td>ERK5</td>
<td>Adipocyte differentiation</td>
<td>Eslau et al. 2004</td>
</tr>
<tr>
<td>miR-1-1 &amp; miR-1-2</td>
<td>Hand2</td>
<td>Cardiac and skeletal muscle formation</td>
<td>Zhao et al. 2005</td>
</tr>
<tr>
<td>miR-181</td>
<td>Hox-A11</td>
<td>Myoblast differentiation</td>
<td>Naguibneva et al. 2006</td>
</tr>
<tr>
<td>miR-101</td>
<td>Unknown</td>
<td>B cell differentiation</td>
<td>Chen et al. 2004</td>
</tr>
<tr>
<td>miR-142s</td>
<td>Unknown</td>
<td>B lymphoid and myeloid differentiation</td>
<td>Chen et al. 2004</td>
</tr>
<tr>
<td>miR-223</td>
<td>Unknown</td>
<td>Myeloid differentiation</td>
<td>Chen et al. 2004</td>
</tr>
<tr>
<td>miR-9</td>
<td>STAT3</td>
<td>Neuronal and astroglial cell differentiation</td>
<td>Krichevsky et al. 2005</td>
</tr>
<tr>
<td>miR-134</td>
<td>Limk1</td>
<td>Dendritic spine development</td>
<td>Schratt et al. 2005</td>
</tr>
</tbody>
</table>

Table 2: Summary of miRNAs Involved in Mammalian Development
In mammals, they have been shown to regulate stem cell fate, developmental and tissue specificity, demonstrating that their expression can be regulated both temporally and spatially, adipocyte differentiation, insulin secretion, B-cell development and they may be important for proper immune function (Esau et al. 2004, Smirnova et al. 2005) and the disregulation of these small RNAs may participate in the pathogenesis of prevalent human diseases, including diabetes, cancer DiGeorge Syndrome, and various virus-related and virus-induced desease (Zhang et al., 2007, Fabbri et al., 2008). MicroRNAs have been shown to regulate the fat metabolism pathway. MiR-14 was the first microRNA reported to be involved in fat metabolism. Inhibiting miR-14 expression results in increased levels of triacylglycerol and diacylglycerol in the fruit fly, Drosophila (Xu et al. 2003). MiR-122, a liver-specific microRNA, is a key regulator of cholesterol and fatty acid metabolism, and is responsible for the up-regulation and down-regulation of several hundred genes (Krutzfeldt et al., 2005, Esau et al., 2006).

By using antagonirs, short RNA molecules designed to silence microRNAs, to inhibit miR-122 in a diet-induced obese mouse, a decrease in plasma cholesterol levels and considerable improvement in hepatic steatosis were observed.

There is also evidence that miR-278 is involved in energy homeostasis in Drosophila (Teleman et al., 2006). Mutants lacking miR-278 are lean. The reason may be that miR-278 mutants have elevated insulin production and sugar circulation mobilized from adipose-tissue glycogen stores. A considerable body of research suggests the potential role of microRNAs as attractive pharmacological agents for metabolic diseases such as obesity. Others were discovered to be important regulator of endocrine functions. The discovery of a pancreatic islet-specific miRNA, miR-375, that inhibits insulin secretion in mouse pancreatic β-cells, uncovered a novel component of the insulin secretion machinery (Fig. 4A) (Poy et al. 2004). miR-375 is believed to act by inhibiting the expression of myotrophin (also known as V-1), a cytoplasmic protein that induces the
exocytosis of insulin granules (Poy et al. 2004). Since type 2 diabetes results from combined defects in insulin secretion and insulin action, miRNAs may have a role in the pathogenesis of this disorder.

Another miRNA that may play a role in endocrine function is miR-143, which has been proposed to play a role in adipocyte differentiation (Fig. 4B) (Esau et al. 2004). Investigators found that reducing the level of this miRNA by transfecting 2′-O-methoxyethyl phosphorothioate-modified antisense RNA oligonucleotides into human pre-adipocytes in vitro inhibited their differentiation, as determined by the decreased expression of 4 adipocyte-specific genes (GLUT4, HSL, fatty acid-binding protein aP2 and PPAR-γ2) and their inability to accumulate triglycerides. Computationally, miR-143 has been predicted to have several targets (Lewis et al. 2003), and the authors determined that knockdown of miR-143 led to upregulation of one of the predicted targets, ERK5/BMK1. ERK5 is thought to promote cell growth and proliferation, which is consistent with the phenotype observed (inhibition of differentiation), but it still remains to be determined whether other targets of miR-143 also contribute to the phenotype.

Since excess adiposity is rampant in western countries, and contributes to several common diseases including type 2 diabetes, hypertension and coronary heart disease, new insights provided by the study of miRNAs in adipocyte biology and long-term energy storage could have a tremendous clinical impact.
Figure 4 | Roles of miR-375 and miR-143.
(A) miR-375 has been shown to be expressed in pancreatic β-cells and is thought to play a role in insulin secretion. In addition, 67 other β-cell-specific miRNAs have been discovered, although their functions have yet to be elucidated. (B) miR-143 has been proposed to have a role in adipocyte differentiation, and inhibition of this miRNA leads to down-regulation of proteins that have a known role in this process (GLUT4, HSL, aP2, and PPAR-γ2 (PPAR-g2)). It is unclear if there are additional miRNAs that are critical for adipocyte differentiation and maturation.
4. Aims of the project

MicroRNA are believed to be generally involved in more quantitative regulation of mammalian trait: therefore, mammalian conditions that have a strong genetic component and involve quantitative differences between the physiological and pathological conditions are strong candidates to be influenced by miRNA disregulations. The pool of this study is thus to investigate the possible role for miRNA imbalance in adipose tissue in the development of obesity.

To date, microRNAs have been identified and evaluated in human preadipocytes (miR-143) and mouse hepatocytes (miR-122). There is no study dealing with microRNA in subcutaneous adipose tissue (SAT) of obese patients.

For this reason, we have performed an high-throughput screening by microarray analysis on 1,458 microRNAs in SAT from 20 morbidly obese patients (10 men and 10 female) versus pools of RNA from non obese subjects (3 men and 5 female).

We were able to identify miRNAs differentially expressed comparing SAT from obese patients with respect to SAT from non-obese patients.

The miR-519d was significantly higher in obese subjects than in the non obese pools.

Using bioinformatic and functional analyses, we have demonstrated that the peroxisome proliferator activated receptor A (PPARA) gene resulted be a target of miR-519d. PPARA regulates the genes involved in fatty acid, glucose metabolism and inflammation (Cho et al., 2008). Therefore, it is conceivable that loss of PPARA could cause metabolic imbalance, thereby resulting in adipocyte hypertrophy in SAT from morbidly obese subjects.

This project set the goal to be a part of the effort in understanding the biology and the role of this miR-519d during biogenesis of obesity.
Materials and Methods
1. Subjects

Subjects were divided into two groups based on body mass index (BMI) used as index of overall adiposity: morbidly obese subjects (BMI>40 kg/m²) and non obese subjects (BMI<30 kg/m²).

The obese subjects included (10 men and 10 women) a subgroup of patients participating in a clinical trial of adjustable gastric banding (Thorne et al., 2002).

The non obese subjects (3 men and 5 women) underwent abdominal surgery because of benign diseases (i.e. laparoscopic cholecystectomy). From these subjects, it was possible to obtain fat specimens from omental as well as subcutaneous adipose tissue (the latter was taken from the surgical incision).

All subjects were Caucasian (Table 3).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Obese (value±SD)</th>
<th>Non-Obese (value±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Age, years</td>
<td>41.1± 3.3</td>
<td>42.2±7.5</td>
</tr>
<tr>
<td>Body mass index, (kg/m²)</td>
<td>42.7 ± 1.2</td>
<td>24.7±1.6</td>
</tr>
<tr>
<td>PL-glucose, mmol/l</td>
<td>5.2 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>PL-cholesterol, mmol/l</td>
<td>5.1 ± 0.3</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>PL-triglycerides, mmol/l</td>
<td>1.4± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>
Eating habits (gorging behavior [70%] and sweet-eaters [30%]), familial history of obesity (30%) and clinical history were recorded for all obese patients. Hypertension was present in 45% of obese patients. Neoplasia, diabetes and alcohol abuse were excluded in all but two subjects: one obese subject was affected by type 2 diabetes and another was addicted to alcohol. All subjects provided written informed consent to the study and the research protocol was approved by the Ethics Committee of our Faculty of Medicine.

2. Samples

Subcutaneous adipose tissues (SAT) were obtained from individual subjects. SAT aliquots (about 100-600 mg), not in contact with abdominal district, were collected during surgery. Electrocauterization was limited to reduce the rate of necrosis. All biopsies were snap-frozen and stored in liquid nitrogen until total RNA isolation and protein purifications. A blood sample was obtained by venipuncture from all fasted subjects the day before the surgery for adipokine analysis and other biochemical measurements.

3. Measurement of adipokines and other biochemical markers

The Bio-Plex suspension array system (Bio-Rad, Hercules, CA), which employs a fluorescent bead based immunoassay, was used to measure adipokine markers of obesity and cytokines: adiponectin, adipsin, ghrelin, leptin, resistin, IL-6 and TNF-α, in serum samples of obese and non obese subjects, according to the manufacturer’s protocol. Briefly, a nine-point standard curve was generated by performing serial dilutions of the reconstituted normalized standard; the assays were run in duplicate. Bioplex manager software was used to analyze data obtained. Main biochemical
parameters (total cholesterol, triacylglycerides, glucose, etc.) were measured by standard methods.

4. RNA extraction

Total RNA was extracted from frozen subcutaneous adipose tissue (SAT) samples by homogenization in guanidine thiocyanate-phenol solution (QIAzol™ Lysis Reagent; Qiagen) and then purified using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol. RNA concentrations and purity were evaluated with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies).

5. MicroRNA expression profile

The expression profiles of microRNAs in SAT were determined using the miRCURY™ LNA miRNA Array V8.1 (208002V8.1-Lot n. 20393.03, Exiqon, Denmark), which contains complementary probes for the mature forms of all microRNAs registered in miRBase 8.1 (Griffiths-Jones et al., 2006). The microarray consist of 1458 capture probes, perfectly matched to human microRNAs annotated in miRBase 8.2, control probes and mismatch probes. The reference RNA consisted of a pool of total RNA isolated from three non obese control males (CM) and five non obese control women (CW) used for the hybridization of obese males and obese women, respectively. Total RNAs of controls were labeled with Cy3 and total RNAs of obese subjects with Cy5 using the miRCURY™ LNA miRNA Array labeling kit following the instruction manual. For each sample, 1 µg of total RNA was labeled hybridized and washed manually using the Agilent microarray hybridization chambers SureHyb.
Microarrays were scanned on an Agilent DNA Microarray Scanner (Agilent Technologies) and raw data were extracted using the Feature Extraction software (Agilent Technologies). Data were subsequently analyzed using GeneSpring® version 7.2 (Agilent Technologies), and statistical analysis was performed using background-corrected mean signal intensities from each dye channel.

Microarray data were normalized using intensity-dependent global normalization (LOWESS).

Differentially expressed microRNAs were identified using a filtering by standard deviation (0-2) and the Benjamini and Hochberg False Discovery Rate (p-value 0.05) to minimize selection of false positives. Of the significantly differentially expressed microRNA, only those with a greater than log₂ 1.5-fold increase or decrease in expression of obese subject compared with non obese controls were used for further analysis. We also used hierarchal gene-tree clustering analysis, and default parameters, to group microRNAs with similar expression profiles.

6. Real-time polymerase chain reaction quantification of selected mRNA

Real-time quantitative PCR was carried out on the Applied Biosystems 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA). cDNAs were synthesized from the same total RNAs utilized in the microarray experiments using hexamer random primers and multiscribe reverse transcriptase (Applied Biosystems). Approximately 1/20th of the resultant cDNA was used for PCR analysis.

We used the oligonucleotides primers for following selected genes:
- SLC2A4 (solute carrier family 2 (facilitated glucose transporter), member 4):
  FW: GCCCCCGCTACCTCTACATC, RW: GCCTGTCAGGCGCTTCA;
- PPARA (peroxisome proliferator-activated receptor alpha):

FW: TTGCTGTGGAGATC, RW: CATGTACAATACCCTCTGCATTT;
- 18S:

FW: CGGCTACCACATCCAAGGAA, RW: GCTGGAATTACCGCGGCT.

The PCR conditions for reverse transcription (RT) were: stage 1: 50°C, 2 min; stage 2: 95°C, 10 min; and stage 3: 95°C, 15s ; 60°C, 1 min/40 cycles.

In parallel, RT-PCR was performed by using the TaqMan miRNA Assay Protocol to assess the relative expression of a subset of microRNAs. The fold changes of mir-519d was normalized to U6 RNA. For RT reactions, 10 ng total RNA was used in each reaction and mixed with the corresponding TaqMan miRNA assays RT primer. The RT reaction conditions were: 16°C for 30 min; 42°C for 30 min; 85°C for 30 min and then hold on 4°C. After the RT reaction, 1.33 µl of cDNA was used for the PCR reaction together with TaqMan primers. The PCR reaction was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

7. Protein extraction and western analysis

Total proteins were extracted from frozen adipose tissue. 200 mg of each sample were homogenized with 1 ml of lysis buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 6.25 mmol/L Tris-HCl pH 6.8 and a cocktail of protease inhibitors (aprotinin 2 mg/ml, leupeptin 2 mg/ml, phenylmethanesulfonyl fluoride 100 mg/ml, pepstatin A 1 mg/ml). Protein concentrations were quantified using the Bradford assay (BioRad Laboratories). For each sample, 50 mg of total proteins were separated by SDS-PAGE (15% for PPARA and SLC2A4) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham) for 19 h at 33 V. Blots were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline tween-20 [(TBST): 25 mM Tris, pH 7.4, 3.0 mM KCl, 140 mM NaCl and 0.1% Tween 20] for 1 h at room temperature. The blots were
incubated with the specific monoclonal antibody; alpha-actinin (dilution 1:1000), PPARA (dilution 1:400); SLC2A4 (dilution 1:200) in the same solution for 1 h. The membranes were rinsed in TBST and incubated for 45 min in TBST buffer with anti-goat IgG, HRP-labeled secondary antibody (dilution 1:5000) for PPARA, SLC2A4 and alpha-actinin.

All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreaction signals were visualized with enhanced chemiluminescence (ECL-PLUS, Amersham Biosciences).

8. Oligonucleotides and plasmids

Chemically modified double-stranded RNA molecules designed to up-regulate (pre-miR-519d microRNA precursor molecules) and down-regulate (anti-miR-519d microRNA inhibitors) endogenous microRNAs were purchased by Ambion (Austin, TX, USA). The plasmids used were pGL3-control encoding for firefly luciferase and pRL-PPARA coding for Renilla luciferase (Promega) modified by insertion of the part of the 3’ UTR region of the PPARA gene containing miR-519d binding sites (1586-2035 bp in the cDNA) in the XbaI site of the pRL-CMV (Promega). The direction of the inserted 3’ UTR region was confirmed by PCR and sequencing after ligation.

9. Cell cultures

The cell lines used were human HEK-293 maintained in α-MEM (minimum essential medium) supplemented with 10% fetal bovine serum and glutamine.
10. Transfection and inhibition experiments

HEK-293 cells were seeded in 24-well plates with 500 µl of antibiotic-free medium the day before transfection to allow adherence and reach 70–90% confluence at the time of transfection. The standard co-transfection mix was prepared for triplicate samples by adding 75 ng pGL3-control, 30 ng pRL-PPARA and pre-miR and anti-miR as indicated in the various experiments in 150 µl of Opti-MEM I (Invitrogen); 3 µl lipofectamine 2000 (Invitrogen) was added separately in 150 µl Opti-MEM I. The two solutions were mixed and incubated at room temperature for 20-30 min, after which, 100 µl of the mix was added to each well. The final volume of medium plus transfection mix was 600 µl. Cells were incubated with the transfection mix for 4 h and the medium was then replaced with new fully supplemented culturing medium. Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured using a dual luciferase assay according to the manufacturer’s instructions (Promega).
Results
1. Identification of miRNAs on subcutaneous adipose tissue through microarray analysis

The recent development of high-throughput gene expression technology permits simultaneous investigation of thousands of genes, providing a snapshot of the transcription state of diseased tissue. Microarray based expression profiling is well suited to investigate the molecular basis of complex diseases such as obesity (Baranova et al., 2005).

In the present study, a microarray screening was carried out on subcutaneous adipose tissue (SAT) in order to identify miRNAs differentially expressed that might be relevant in the development of obesity.

In order to analyze the miRNA expression profiles we used microRNA microarrays that contain high affinity LNA-based capture probes for all human microRNAs in the release 8.2 of the miRBase microRNA Registry (http://microrna.sanger.ac.uk/).

LNA (Locked Nucleic Acid)-modified oligonucleotides have been used as sensitive and specific miRNA detection probes (Castoldi et al., 2006). Incorporation of LNA into the probes increases the affinity for the complementary miRNA targets. This LNA-array captures probes are therefore more sensitive than DNA probes and are designated with a uniform Tm that guarantees that each miRNA hybridizes to the array with equal affinity and specificity. LNA array consists of control probes snRNAs (U6), that are commonly user for normalization, mismatch probes, a non-target capture probe and target capture probe. Several of the capture probes included are designed for synthetic target that do not occur biologically.

We used 1458 different capture probes to evaluate the different expression of microRNAs in adipose tissue from two groups of subjects. The first group is composed
of 10 obese woman (OBW) and 10 obese men (OBM); all subjects were extremely obese with a BMI $\geq 40$ and undergoing surgery to reduce body weight.

The second group is composed of 3 non-obese man (CM) and 5 non-obese woman (CW) controls with a BMI $\leq 30$ and normal adipose tissue distribution that underwent surgery for others conditions.

We extracted, from samples of subcutaneous adipose tissues from each subject, RNA and proteins.

We chose RNA from subcutaneous because its uniform tissue composition and absence of immune system cells that are, on the other hand, abundant in omental fat (Linder et al., 2004).

Total RNA has been purified and quantified and only high quality RNA was used for the screening. Subcutaneous adipose tissue RNA of obese patients was labelled with fluorochrome Cy3 while adipose tissue RNA of non-obese patients was pooled and labelled with fluorochrome Cy5 served as controls.

Expression profiling revealed that a large set of microRNAs was expressed in SAT. Forty-two microRNAs differed by at least log$_2$$\pm$1.5 fold (p-value$<$0.05) in 17/20 obese subjects versus the non obese control pools. Of the forty-two microRNAs differed, 21 were up-regulated and 21 were down-regulated. Among the differentially expressed microRNAs, in 20/20 obese subjects, three microRNA were up-regulated (Fig. 5), whereas two microRNA were down-regulated (Fig. 6).

Our attention was focused on the up-regulated miR-519d, the microRNA significantly higher in obese subjects than in the non obese pools.
Figure 5 | Up-regulated microRNAs in 20/20 severely obese subjects.
MicroRNA expression profiles obtained from LNA microRNA Arrays V 8.1 of subcutaneous adipose tissue from obese men and women. The rows represent different microRNA probes on the microarray, and the columns represent individual RNA samples. The scale at the top indicates relative microRNA expression changes in log2 units, where 0 represents the mean expression level of a given microRNA across samples.
Figure 6 | Down-regulated microRNAs in 20/20 severely obese subjects.
2. Real-Time PCR

In order to confirm the results of microarray analysis, microRNAs expression from the same samples from obese and non-obese groups were analyzed by Real-Time PCR assay.

Real-Time PCR assay is a highly sensitive approach for identifying miRNA profiles (Chen et al., 2005). For this assay, we have used stem–loop RT primers rather than conventional linear ones, because of their higher specificity and sensitivity likely due to the base stacking and spatial constraint of the stem–loop structure. The assays target only mature microRNAs, not their precursors, ensuring biologically relevant results.

In almost all patients, microarray profiling data correlated with real-time RT-PCR data (Fig. 7). In fact, the expression levels of miR-519d were confirmed in OBW and in OBM versus CW and CM, respectively.

3. Identification of miRNA target

Computational algorithms have been the major driving force in predicting miRNA targets (Rajewsky, 2006). These approaches are mainly focused on programming alignment to identify complementary elements in the 3’-UTR with the seed sequence of the miRNA and the phylogenetic conservation of the complementary sequences in the 3’-UTRs of orthologous genes. However, evidences suggest that perfect seed pairing may not necessarily be a reliable predictor for miRNA interactions (Diadian and Hobert, 2006), which may explain why some predicted target sites are non functional. Hence, with few exceptions, most physiological, and clinically relevant, targets for miRNAs remain to be identified or verified experimentally. Currently there is no clear consensus as to what criteria should be followed to determine miRNA targets and to confirm their
Figure 7 | microRNA expression levels in subcutaneous adipose tissue from morbidly obese subjects.
MiR-519d expression levels are very high in morbidly obese women and men, respectively.
A: obese women [OBW], CW, control women pool; B: obese men [OBM], CM, control men pool.
Data are expressed as RNA fold change in obese patients versus non obese control pool (RQ=2^[deltaCt]).
biological efficacy. A set of guidelines has been established to validate the hypothesis that a given miRNA regulates a specific mRNA target as showed in the following scheme (Fig. 8).

A predetermined gene as a target of miRNA regulation, individual gene sequences (i.e. 3'-UTR mRNA sequences) may be analyzed by various computational algorithms which utilize distinct parameters to predict the probability of a functional miRNA binding site within a given mRNA target.

There are three bioinformatic algorithms, used in different applications, that are capable to predict miRNA target sites: miRanda (http://microrna.sanger.ac.uk) (Griffiths-Jones et al., 2006); TargetScan (http://www.targetscan.org) (Grimson et al., 2007); and, PicTar (http://pic.tar.bio.nyu.edu) (Krek et al., 2005). All these computational programs allow the investigator to enter a specific ‘Gene Symbol’ and the algorithm will compute all predicted miRNA target sites within that gene. Additionally, these algorithms will determine all the putative mRNA targets of a given miRNA.

To further reduce the number of putative miRNAs that may repress the expression of a given mRNA target is necessary further in silico analysis since many miRNA targets predicted by seed sequence matching often fail validation tests in vivo (Diadian and Hobert, 2006). Contextual features may also govern miRNA/mRNA interactions. For example, a large portion of a given mRNA sequence is highly structured and only certain single-stranded regions may be accessible for binding with miRNAs. Thus, complex RNA secondary structures may prevent miRNA/ mRNA interactions; in fact, a common feature of most validated targets is that miRNAs preferentially target 3'-UTR sites that do not have complex secondary structures and are located in accessible regions of the RNA based on favourable thermodynamics (Zhao Y et al., 2007). Since RNA accessibility may be a critical feature of miRNA target recognition and the free energy
Figure 8 | Proposed flow diagram microRNA validation
(ΔG) of the 70 nucleotides flanking the 50 and 30 sides of the predicted miRNA binding sites is determined using mFold program (http://mfold.bioinfo.rpi.edu/).

All target genes were analyzed by TargetScan (release 4.2) because it evaluates various features of microRNA/mRNA interactions, and targets are ranked by ‘total context score’, which is based on site type, site number and site context.

The list of predicted up-regulated targets gene and pathways of the hypothetical targets of miR-519d are showed on line (http://www.targetscan.org/cgi-bin/vert_42/targetscan.cgi?mirg=hsa-miR-519d).

4. Identification interesting pathway

The lists of gene target with a favourable context score (<-0.30) were combined and organized by KEGG GENES Database (http://www.genome.jp/kegg/). KEGG is a collection of gene catalogs for all complete genomes and some partial genomes generated from publicly available resources, mostly NCBI Ref Seq. It integrates current knowledge on molecular interaction networks such as pathways and complexes, information about genes and proteins generated by genome projects and information about biochemical compounds and reactions. The role of adipose tissue as a dominant regulator of whole-body lipid and glucose homeostasis is now well established, based on extensive experimental evidence, which shows that dysfunctions in adipose tissue metabolism have a direct impact on lipid and glucose homeostasis. Many observations reinforce the concept that normal lipid and glucose homeostasis as well as normal insulin sensitivity requires fully functional adipose tissue. Adipose dysfunctions in obesity include secretions of abnormal levels of cytokines linked to insulin resistance, impairments in triglyceride storage and increases in lipolysis (Guilherme et al., 2008).
From these foundations, interestingly, were tried, among those identified two very interesting pathways, own those indicated over which are often involved in obesity and obesity-associated diseases: insulin and adipocytokine signaling pathways.

The following genes were selected from these two pathways:

- **PPARA** = target of miR-519d with 5 binding sites
  
  (1:7mer, which is highly conserved, and 3:7mer and 1:7mer-1A, which are poorly conserved)

- **SLC2A4** = target of miR-519d with only 1 binding site
  
  (7mer-m8 highly conserved)

All these genes have a good microRNA accessibility (ΔG analysis) evaluated with the mFold program (http://mfold.bioinfo.rpi.edu/) and a favourable context score.

From bioinformatics predictions may predict a large number of putative miRNA binding sites on a specific targets, but a rapid and reproducible assay is needed to quickly evaluated interaction sites are functional.

### 5. RT-PCR and Western-Blot

The mRNA expression of these putative target genes in SAT from obese and non-obese subjects was verified by RT-PCR (Fig. 9). The levels of SCL2A4 did not differ between obese and non obese subjects whereas the levels of PPARA were particularly high in OBM (mean RQ±SD: 6.5±3.0) versus respectively CM and CW. The effects of up-regulation of microRNA-519d are obvious by Western-Blotting. We chose 2 OBW and 2 OBM who had different levels of microRNA-519d to determine a dose-dependent effect on target protein. Protein concentrations of SLC2A4, is similar in the obese and non obese subjects, whereas the levels of PPARA protein are different and the data suggests that PPARA plays a relevant role in the development of obesity (Fig 10).
Figure 9 | Gene expression analysis of selected genes in subcutaneous adipose tissue from morbidly obese subjects.
Predicted targets of up-regulated miR-519d in obese subjects are SCL2A4 and PPARA were analyzed by RT-PCR.
A: obese women [OBW], CW, control women pool; B: obese men [OBM], CM, control men pool.
Figure 10 | PPARA is down-regulated in subcutaneous adipose tissue from morbidly obese subjects. Western-blot analyses of selected predicted targets SCL2A4 and PPARA of up-regulated miR-519d in obese subjects.
6. Luciferase assay

A common experimental reporter system able to verify predicted miR/mRNA pairings, is luciferase reporter assays.

The rationale for using this assay is that the binding of a given miRNA to its specific mRNA target site will repress reporter protein production thereby reducing activity/expression that can be measured and compared to a control.

The experimental approach is to clone the 3’-UTR of the target gene of interest, or (repeated) fragments thereof, immediately downstream of the luciferase measuring the expression of the construct in transfected cells.

3’-UTR of PPARα is composed of about 8 Kb of nucleotides and presents five miR-519d binding sites, one conserved and four poorly conserved.

The plasmids used were pGL3-control encoding for firefly luciferase, a control luciferase reporter plasmid, and were co-transfected with pRL-PPARA, coding for Renilla luciferase under CMV promoter, including two miR-519d binding sites (one conserved and one poorly conserved) on five contained in a part of the 3’ UTR region of the PPARA gene. The direction of the inserted 3’ UTR region was confirmed by PCR and sequencing after ligation.

7. Validation of the interaction between miR-519d and the PPAR-alpha 3’UTR

It is possible to test effects of the miR-519d transfecting the chemically synthesized miR into cells together with the reporter plasmid harboring multiple, concatenated binding sites designed to mimic the mismatched base pairing typical of known endogenous miR/mRNA pairings.
Previously, 2’-O-methyl oligonucleotides have been demonstrated to mediate inhibition of miRNAs in vitro (Meister et al., 2004) and in vivo (Leaman et al., 2005) and has found wide use as miRNA inhibitors. Recently, LNA-modified oligonucleotides can mediate this inhibition of miRNAs equally efficient way. LNA has features and results in very high hybridization affinity towards complementary single stranded RNA without compromising specificity and show improved antisense efficacy and higher Tm toward complementary RNA compared to 2’-O-methyl oligonucleotides of the same sequence (Ørom et al., 2006).

From these premises, LNA-modified oligonucleotides anti-miRNA and pre-miRNA were used to verify that miRNA activity with luciferase assay.

Successively, we transfected another cell line, HEK 293, that have an higher efficient of transfection than precedent cell line (Chub-S7) (Fig. 11).

A related approach, with certain advantages as outlined later, is to synthesize target mRNAs by in vitro transcription and transfect cells directly with an mRNA/miR mix.

After transfection into HEK-293 cells of the pRL-PPARA plasmid with pre-mir-519d, who mimics the function of the same microRNA, in increasing concentrations (100nM, 200nM e 300nM). We observed an inhibition of luciferase activity of about 50% compared to pRL-PPARA plasmid transfection alone (Fig. 11a). Addition of premiR-519d was able to promote the repression of luciferase activity in a concentration-dependent manner.

In a second experiment, after transfection into HEK-293 cells of the pRL-PPARA plasmid with only one concentration of pre-mir-519d (200nM), we inhibited endogenous pre-mir activity using antisense oligonucleotides, antimir-519d, complementary to the same microRNA, in increasing concentrations (100nM and 200nM).
We observed a block of the repression of *luciferase activity* compared to pRL-PPARA plasmid transfection with the premir-519d (200nM) (Fig. 11b).

This data demonstrates that there is an effective, specific and functional interaction between miR-519d and the 3’UTR of the PPARA mRNA suggesting that changes in miR-519d levels may effectively modulate the expression of genes regulated by this transcription factor.

Application of this method makes the further assumption that miRNA-mediated repression results from a graded reduction in translation of all functional mRNA molecules, rather than a complete translational shutdown of a subpopulation thereof.
Figure 11 | MiR-519d binds and functionally interacts with PPARA 3’UTR.

(A) pre-miR-519d dose-dependently inhibited the expression of a Renilla luciferase-expressing construct that contains the 3’ UTR region of the PPARA gene containing miR-519d putative binding sites; (B) anti-miR-519d dose-dependently blocked the pre-miR-519d-induced inhibition of the expression of Renilla luciferase containing the PPARA 3’UTR region. Each sample, except the positive control, was transfected with a 200 nM final concentration of pre-miR-519d and the indicated concentration of the anti-miR-519d. *P<0.05; **P<0.01.
Discussion
A growing body of evidence suggests that microRNAs are important regulators of cell growth (Ambros, 2004), differentiation (Hwang et al., 2006), and apoptosis (Wienholds et al., 2005). In support of the significance of miRNAs in normal development and physiology, recent mouse miRNA “knockout” studies demonstrated that depending upon which miRNA gene was deleted, mice were left immuno-deficient (Thai et al., 2007) or with heart defects (Zhao et al., 2007). Implications drawn from these studies support the hypothesis that dysregulation of miRNA function may lead to human disease. As disease-specific miRNAs are identified, the validation of novel targets within a disease pathway of interest may lead to novel therapeutic strategies.

Therefore, it is critically important to be able to identify and validate miRNA/mRNA target pairs. Although not perfect, computational algorithms and ΔG analyses allow for the identification of putative miRNA/mRNA targets. Once identified, the authenticity of a functional miRNA/mRNA target pair can be validated by fulfilling criteria. First, miRNA/mRNA target interaction must be verified. Second, the predicted miRNA and mRNA target gene must be co-expressed. Third, a given miRNA must have a predictable effect on target protein expression. That is, if gene is a true target of a given miRNA, its miRNA mimic will decrease the target gene expression level while a miRNA inhibitor will increase the target gene expression level. Fourth and final, miRNA-mediated regulation of target gene expression should equate to altered biological function.

There is currently no consensus model for the mode by which miR affect the process of mRNA translation. Few studies have, so far, addressed this possibility. Detailed biochemical characterization of the miR mechanism will depend on the development and application of tractable cell-free translation systems that preserve the key physiological features of their action. Further, it may be just as instructive to determine “where” in the cell miR-dependent translational repression takes place, as to
investigate “how” it happens. An important task will also be to attempt independent verification of key findings that led the different investigators to propose their divergent models (Clancy et al., 2007).

This work reports for the first time an high-throughput screening by microarray analysis on microRNA expression profiling on human subcutaneous adipose tissue from obese and lean subjects to improve our understanding of global transcriptional regulation in severe obesity. A global analysis of miRNA expression pattern identified five miRNAs involved in obesity. From this study the miR-519d was significantly higher the in obese subjects than in the non obese pools. PPARA was predicted to be a miR-519d target by five binding-sites on the PPARA 3’ UTR. Interactions on each sites act independently and aren’t cooperative (Grimson et al., 2007). We found PPARA protein levels down-regulated in obese subjects than in the non obese pools. It may be that miR-519d acts through different molecular mechanisms but we were able to validate experimentally the interplay between miR-519d and PPARA in a context-dependent manner.

PPARA was the first PPAR to be identified and is highly expressed in tissues that rely on fatty acid oxidation as their primary energy substrate, namely, heart, liver and skeletal muscle (Braissant et al., 1996).

Nuclear receptors, especially peroxisome proliferator-activated receptor-alpha (PPAR alpha) is a ligand-activated transcription factors that regulate the metabolism of glucose and genes encoding fatty acid oxidation enzymes. PPAR-alpha is necessary for the induction of peroxisomal biogenesis in response to peroxisome proliferators. Most known PPAR-alpha target genes encode enzymes involved in cellular fatty acid oxidation including the peroxisomal, mitochondrial, and cytochrome p450 pathways. PPAR-alpha is activated by fatty acids or inhibitors of mitochondrial long-chain fatty acid import. These data, taken togheter suggest that PPAR-alpha serves as a cellular
'lipostat', transducing changes in cellular lipid levels to the transcriptional regulation of target genes involved in fatty acid utilization.

To test the hypothesis that PPAR-alpha is activated as a component of the cellular lipid homeostatic response, Djouadi et al. (1998) characterized the expression of PPAR-alpha target genes in response to a perturbation in cellular lipid oxidative flux caused by pharmacologic inhibition of mitochondrial fatty acid import. Inhibition of fatty acid oxidative flux caused a feedback induction of PPAR-alpha target genes encoding fatty acid oxidation enzymes in liver and heart. In mice lacking PPARA (Ppara -/-), inhibition of cellular fatty acid flux caused massive hepatic and cardiac lipid accumulation, hypoglycemia, and death in 100% of male, but only 25% of female Ppara -/- mice. The metabolic phenotype of male Ppara -/- mice was rescued by a 2-week pretreatment with beta estradiol. These results demonstrated a pivotal role for PPAR-alpha in lipid and glucose homeostasis in vivo and implicated estrogen signaling pathways in the regulation of cardiac and hepatic lipid metabolism.

Studies in rodents demonstrated the involvement of PPAR-alpha nuclear receptor in lipid homeostasis, with a sexually dimorphic control of circulating lipids, fat storage, and obesity (Costet et al., 1998).

Adenovirus-induced hyperleptinemia causes rapid disappearance of body fat in normal rats, presumably by upregulating fatty acid oxidation within white adipocytes. To determine the role of PPARA expression, which was increased during the rapid loss of fat, Lee et al. (2002) infused adenovirus-leptin into Ppara-null and Ppara-wildtype mice. Despite similar degrees of hyperleptinemia and reduction in food intake, epididymal fat pad weight declined 55% in wildtype but only 6% in null mice; liver triacylglycerol fell 39% in the wildtype group but was unchanged in the null group. Carnitine palmitoyltransferase-1 rose 52% in the wildtype mice but did not increase in the null mice. The most striking transcription difference was the 3-fold rise in PGC1-
alpha (PPARGC1) in white adipose tissue that occurred in Ppara-wildtype but not in Ppara-null mice. Moreover, baseline expression of PGC1-alpha in the null mice was below normal. The role of the PGC1 coactivator in mitochondrial biogenesis, thermogenesis, and gluconeogenesis is well established. Lee et al. (2002) found the most plausible interpretation of the findings in white adipose tissue to be that leptin induces, through upregulation of PGC1-alpha expression, a PPARA-dependent increase in mitochondrial biogenesis that increases fatty acid oxidation sufficiently to deplete triglyceride stores with a relatively modest increase in the transcription and/or activities of the enzymes of fatty acid oxidation. Hypertension and diabetes are common side effects of glucocorticoid treatment. Human hepatocytes treated with dexamethasone and a PPAR-alpha agonist showed induction of PPARA and gluconeogenic gene expression. Kintscher et al. have shown the increased expression of PPARA (measured as mRNA level) in overweight (BMI 29±3 kg/m²) as compared with normal weight patients. They suggested that characteristic for obesity and metabolic syndrome elevation of FA in blood leads to permanent PPARA stimulation, since free fatty acids are ligands for these transcription factors and itself activates its expression. PPARA activation may lead to the stimulation of beta-oxidation and this process turns into lipotoxicity, and cardiomyopathy parallel with development of obesity (Kintscher et al., 2003).

The present work has led to the identification of an “interactome” network involved in the pathogenesis of obesity. We have shown that integration of microRNA-protein expression and clinical data can be used to generate a network of potential functional associations with obesity and alterations in gene expression are able to perturb this relationship. This is in line with our results indicating that in human obesity miR-519d-mediated repression of the PPARA. Taken together, the data obtained in animal models show that PPARA plays a relevant role in lipid storage in adipose tissue and increases adiposity. The results will help to improve the understanding of the
pathogenesis of multifactorial diseases such as obesity and provide possible novel therapeutic targets.
References


