MicroRNA-196 Regulates HOX Gene Expression in Human Gluteal Adipose Tissue

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Objective: Lower body fat is associated with diminishing cardiometabolic risk. Physiological differences between gluteofemoral and abdominal subcutaneous adipocyte functions are known, but the molecular basis for depot differences in adipocyte function is poorly understood. The objective of this study was to identify depot differences in microRNA (miRNA) expression in human abdominal and gluteofemoral subcutaneous adipose tissues and their implication in gene regulation.

Methods: Abdominal and gluteofemoral adipose tissue aspirates obtained from 18 participants (9 male and 9 female, age 30 ± 1.5 y, BMI 27.3 ± 1.23 kg/m²) were analyzed for miRNA expression profiles by next-generation DNA sequencing. The raw reads were mapped to miRBase 17, and differentially expressed miRNAs were confirmed by qRT-PCR. The hsa-mimic-miR196a was transfected into cultured abdominal preadipocytes isolated from five women with obesity. Target gene expression was evaluated by RT-qPCR.

Results: Among the 640 miRNAs detected in adipose tissue, miR196a2, miR196a1, miR196b, and miR204 showed a higher expression in the gluteofemoral depot (fold change = 2.7, 2.3, 1.7, and 2.3, respectively) independent of sex. Bioinformatic analyses and human primary preadipocyte transfection with miR196 suggested that the differentially expressed miRNAs could directly or indirectly modulate homeobox (HOX) gene expression.

Conclusions: The miR196 gene family could play an important role in the regulation of HOX gene expression in subcutaneous adipose tissue and in fat distribution variation.

Introduction

Overweight and obesity present a worldwide clinical and public health burden and are correlated with an increased risk of cardiometabolic diseases, cancer, and mortality (1,2). In individuals with obesity, triglycerides are stored in an expanded adipose tissue, leading to inflammation, altered adipokine secretion, and inflexible lipid metabolism. However, not all individuals with obesity have the same risk of developing these complications. Multiple correlation studies have shown that central obesity, characterized by triglyceride accumulation in visceral and abdominal subcutaneous depots, is closely associated with the development of cardiovascular disease and type 2 diabetes (3,4). Inversely, lower body fat, defined as fat accumulation in the gluteofemoral (GF) region and typically observed in premenopausal women, appears to be protective and is paradoxically associated with improved metabolic and cardiovascular profiles (3,5-7). GF body fat distribution plays a key protective role in metabolic disorders associated with overweight and obesity; therefore, understanding the mechanisms regulating its expansion is an important challenge. Excellent reviews showing basic physiological differences in abdominal and GF adipocytes have been recently published, mainly focusing on gender specificity (8-12). However, we currently do not understand the underlying molecular basis for depot differences or the molecular mechanisms that control the development and function of the different subcutaneous adipose tissues.

At the transcriptional level, previous transcriptome profiling in our laboratory found that genes in the homeobox (HOX) family were
MicroRNAs (miRNAs) represent the most abundant regulators of gene expression in the human genome. Through their influence on target messenger RNAs (mRNAs), miRNAs are involved in numerous physiological and pathological processes and, in particular, in many functional aspects of adipocyte differentiation (15), and they potentially contribute to the pathogenesis of obesity (16,17). Some of the more interesting genomic locations of miRNA genes include those in the HOX clusters, where they can affect the translational repression of the HOX genes but could also mediate degradation of the target mRNA (18).

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As part of a broader exploration to identify the molecular mechanisms underlying the fundamental differences in GF and abdominal subcutaneous adipose tissue, we hypothesized that a differential miRNA profile exists between abdominal and GF adipose tissue depots. We employed a new DNA sequencing technology to identify miRNAs present in abdominal and GF subcutaneous human adipose tissue. This approach allows the sequencing and direct quantification of all the miRNAs in a sample and should complement the previous work of Rantalainen et al., who analyzed global miRNA expression in GF and abdominal adipose tissue (13). Finally, we tested these potential interactions in vitro by transfecting primary human preadipocytes with a pre-miRNA that mimics endogenous precursor miR196a.

### Methods

Twenty-one men and fourteen women were recruited according to the criteria described previously (13). To participate, volunteers needed to be between the ages of 18 and 40 with 20% to 50% body fat. At screening, volunteers were excluded for significant medical illness, glucocorticoid use, smoking, or substance/alcohol abuse, and women needed to be in a normal menstrual cycle. Oral contraceptive pills were not allowed. Men and women were matched for age, ethnicity, and BMI. The original study was a multicenter translational research protocol conducted at three sites (University of Maryland, Baltimore, Maryland; Pennington Biomedical Research Center, Baton Rouge, Louisiana; and University of California San Diego, San Diego, California) after approval by the appropriate Institutional Review Boards. According to tissue availability, we selected nine men and nine women for the conduct of our substudy. The characteristics of the substudy population are presented in Table 1.

After an overnight fast, blood samples were collected, accessioned, aliquoted, frozen, and then shipped to the Pennington Biomedical
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Research Center Clinical Chemistry Laboratory for analysis. Body composition was measured by dual-energy X-ray absorptiometry using a single brand of the instrument (Hologic QDR 4500A, Hologic, Waltham, Massachusetts).

Adipose tissue biopsies were collected, and total RNA was isolated as described previously (13).

miRNA sequencing

We used the SOLiD Total RNA-Seq Kit (Thermo Fisher Scientific, Waltham, Massachusetts) for preparing small RNA libraries from abdominal and GF adipose tissue and the SOLiD 4 next-generation sequencing instrument to identify the miRNAs. Briefly, the small RNA (20-45 nucleotide region) was enriched from total RNA using flashPAGE gel and was concentrated by precipitating overnight with sodium acetate and ethanol. The small RNA was used as input for library preparation as described in the protocol “SOLiD Small RNA Expression Kit protocol” (part# 4399434, Applied Biosystems). The detection method was based on sequencing by ligation. The 40 libraries were multiplexed and loaded onto two full slides, and 35-bp reads were generated from the SOLiD 4 instrument.

miRNA sequence data analysis

The raw SOLiD reads contained part of the adapter sequences, as the SOLiD reads (35 nucleotides) were longer than most of the expected length of mature miRNAs. The “cutadapt” program was first applied to trim the adapters from the reads (21). Trimmed reads that were shorter than 15 nucleotides were discarded. Subsequently, reads that matched RNA contaminants such as tRNA, rRNA, and DNA repeats were filtered out. The remained trimmed reads were mapped to the human genome (hg19; UCSC Genome Browser) and to the known mature miRNAs (miRBase, release 17) using the Bowtie aligner (22) with parameters (-n 0 –l; 16 –e; 100 –m 5; –best). The mature miRNA coordinates in hg19 were based on miRBase release 17 (23). The raw expression values (read counts) were obtained by summing the number of uniquely mapped reads to mature miRNAs or to mature miRNA coordinates. Normalized expression values were obtained using the reads per kilobase per million approach (24). Custom Perl scripts were developed to summarize the raw read counts and normalize the expression values.

Statistical analysis of the miRNA sequencing data

Genes with zero read counts across all samples were filtered from the database. Box plots and M-A plots were applied to evaluate the data quality for each replicate. Two samples that showed extremely low counts were excluded. After cleaning the data, the read counts were logarithmically (base 2) transformed and then used for downstream analysis. Accounting for the high proportion of zero and low counts of each sample, the upper-quartile (3rd) normalization method was applied to remove any impacts of technical factors and sequencing depth among samples. Specifically, a negative binomial general linear model was used to detect the statistical differential genes between two depots. The raw P values generated from multiple comparisons were adjusted by false discovery rate (FDR) using the linear step-up method of Benjamini Hochberg. A total of 2,640 hypotheses were tested. A miRNA was considered to be significantly different between two depots if the FDR-adjusted P value was less than 0.05 and fold change of expression was greater than 1.3 in either direction. All statistical analyzes were performed in SAS version 9.2.

Confirmatory miRNA gene expression analyses

miRNAs were reverse transcribed by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). miRNA expression was assessed by real-time polymerase chain reaction (PCR) using a Viia 7 sequence detection system (Life Technologies, Carlsbad, California) and TaqMan technology suitable for relative miRNA expression quantification using the following parameters: one cycle of 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 1 minute. For all assays, the RNU48 gene was used as internal control. All expression data were normalized by dividing the target gene by the internal control.

Human adipocytes and SVF isolation

Adipocytes and SVF were isolated by collagenase digestion with 1 mg/mL collagenase type 1 (Worthington Biochemical, Lakewood Township, New Jersey) in Hanks’ balanced salt solution shaken for 2 hours at 37°C and used for RNA extraction.

Preadipocyte culture and miRNA precursor transfection

Human preadipocytes were isolated from the abdominal adipose tissue biopsies from women with obesity and cultured as described previously (13). One week after seeding, 5 nM of hsa-miR-196a-5p mimic (mimics the all miR196 family including miR196a1, miR196a2, and miR196b) or negative control (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts) were transfected into abdominal preadipocytes using lipofectamine RNAiMax (Life Technologies, Carlsbad, California) for 72 hours. At the end of the transfection, the number of viable cells was measured using the RealTime-Glo MT Cell Viability Assay (Promega Corp., Madison, Wisconsin). Experiments were conducted in cells isolated from five different donors (age 30 ± 6.9 years; BMI = 34.6 ± 2.2 kg/m²; waist-to-hip ratio = 0.91 ± 0.04), and experiments were performed in duplicate.

Bioinformatics analysis: pathways and structural equation model mapping

The pathway analysis was performed with Ingenuity Pathway Analysis (IPA), using a new miRNA target filter functionality that enables prioritization of experimentally validated and predicted mRNA targets. Data input were the HOX genes and the miRNAs differentially expressed between both depots. The network pathways and biological functions were tested by Fisher’s exact test. All significant differential genes (mRNA and miRNA) were highlighted with green (>1) and red (GF/Abdominal > 1) in Supporting Information Figures S1-S2.

A modified structural equation model (SEM) algorithm was applied to construct the coexpression network of miRNA and mRNA. It included two stages: P-stage and S-stage. In the P-stage, partial least square regression was used to select a cluster of new genes with high correlations to target mRNA. Then, in S-stage, SEMs were performed to build relationships between this cluster of miRNA genes to target mRNA based on multivariate multilayer linear regression coefficients. This two-stage SEM analysis was performed in SAS version 9.2.
Results

Identification of miRNAs differentially expressed in the GF depot

Abdominal and GF adipose tissue aspirates obtained from premenopausal women and age- and BMI-matched men were used for identifying miRNAs by SOLiD sequencing technology. Their demographics and clinical characteristics are shown in Table 1. Among the 1,733 known miRNAs, 640 were present in the subcutaneous adipose tissue, defined as present only in an abdominal depot, only in a GF depot, or in both depots. Thirty-seven miRNAs (2.1%) were differentially expressed between abdominal and GF adipose tissue, 25 being significantly downregulated in GF adipose tissue, whereas 12 were significantly upregulated. Figure 1 presents the fold change (FC) of all 37 miRNAs differentially expressed (\(P\) value < 0.05), with a maximal FC of 24.3 and a vast majority of miRNAs (\(n = 30\)) showing an FC between 1.37 and 3.40.

Because the expansion of lower-body subcutaneous depots is associated with protection from impairments in glucose-insulin homeostasis and hypertriglyceridemia, we chose to focus on the miRNAs upregulated in the GF depot, developing the hypothesis that they have a role in GF tissue development. Among these genes, only miR196, miR203, miR204, miR205, and miR3545 showed an expression detectable by quantitative PCR. miR196 family genes also showed a very interesting genomic location, in the middle of HOXA, HOXB, and HOXC clusters. miR205, miR204, and three isoforms of miR196 (miR196a1, miR196a2, and miR196b) showed an increase in GF adipose tissue (FC 21, 2.3, 2.3, 2.7, and 1.7, respectively; Figure 1), without distinction between males and females. No significant difference appeared for these four genes between females and males, no matter the depot (Figure 2). Inversely, miR205 was highly expressed in male compared to female adipose tissue in both depots, FC 12.4 for the abdominal depot and 154 for the GF depot (Figure 2).

miR203a and miR3545 (or miR203b), located at the same region on chromosome 14, were specifically increased in GF tissue compared to the abdominal depot only in males (five- and sixfold, respectively; Figure 2) with no difference in female adipose tissues. These two miRNAs showed a significant increase in the male GF depot compared to the female GF depot (12.7- and 13.6-fold increase respectively; Figure 2), with no difference between sexes in the abdominal depot.

qRT-PCR confirmation

To confirm these sequence-based differences, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on the same isolated RNA using specific primers for the corresponding mature form of miRNA. miR196a (primers recognizing miR196a1 and miR196a2) and miR204 were significantly higher expressed in the GF depot compared to the abdominal depot (Figure 3A). We observed the same tendency for miR196b (\(P = 0.07\)), but no difference was detected between depots for miR205, miR203, or miR3545.

To gain insight into the causes of the differences in miRNA expression, we analyzed the association of selected mature miRNAs of interest with clinical/anthropometric variables. This analysis was performed with gene expression data in both depots. As shown in Figure 3C, the correlations between percent body fat, waist circumference, and the expression of miR196a1 and miR204 were found only in the abdominal depot. Inversely, the correlation between hip circumference and miR196a2 expression was found only in the GF depot (Figure 3C).

A second independent group of healthy participants (\(n = 2\) F/2 M; age = 27 ± 1.84 years; BMI = 28.4 ± 2.53 kg/m²; waist-to-hip ratio = 0.87 ± 0.04) was recruited to address the question of whether the differential expression of the miRNAs previously observed is due to

Figure 1 MicroRNAs differentially expressed between abdominal and GF subcutaneous adipose tissue in both sexes (FDR \(P < 0.05\)). Fold change is the ratio between abdominal and GF expression. Black bars indicate microRNA selected for qRT-PCR validation. Fold changes were calculated by dividing the average of the means of miRNA copies number in abdominal depot by the average of the means of miRNA copies number in the GF depot.
altered in adipocytes and/or the SVF. As shown in Figure 3B, miR196a, miR196b, and miR204 are expressed in both collagenase-isolated adipocyte and SVF. Depot differences were apparent in both fractions (except for miR196a in SVF). The tendency was nevertheless more apparent in adipocyte fractions (Figure 3B). For these selected miRNAs, expression differences observed in adipose tissues were not maintained in culture after differentiation of human preadipocytes (data not shown), i.e., they were not cell-autonomous.

Identification of miRNA-HOX gene interactions by pathway analysis and SEM

We recently identified several HOX genes downregulated in the GF depot of both sexes, and we hypothesized that these genes might play a role in the interindividual variation in fat distribution. To identify gene networks connected to specific miRNAs that were higher in GF adipose tissue, we performed IPA. These analyses regrouped the miRNAs differentially expressed between depots into two different modules (Supporting Information). Cellular development, tissue development, cellular growth, and proliferation were identified as the primary functions for genes implicated in the first network of genes. Interestingly, pathway analysis highlighted a direct interaction between miR204 and HOXA10, a mRNA that we previously identified as strongly upregulated in GF male and female adipose tissue (13).

The second module established a relationship between miR196a/b and a different set of HOX genes (Supporting Information Figure S2), characterized by direct interactions between miR196a/b and HOX8 genes (HOXB8, HOXC8, HOXD8), three mRNAs that are downregulated in GF depots. IPA also identified direct interaction between miR196a/b and HOXA7 and indirect interactions with HOXB7.

We then performed correlation analysis using SEM, described in details in Methods and reference (25). The HOX gene expression data, previously obtained by transcriptome microarray experiment with the same group of subjects (13), were loaded into the model along with each miRNA. To study only the relationships between genes showing a differential expression between both depots, the correlations were established between the ratio of expression in abdominal depots and in GF depots. We identified gene expression

![Figure 2](image-url) miRNA sequencing quantification for three selected microRNAs differentially expressed between abdominal (white bars) and gluteal (grey bars) subcutaneous adipose tissue split by sex. Data are presented as mean ± SEM of females (n = 9) and males (n = 9). p < 0.05, female vs. male. p < 0.05, gluteal vs. abdominal. GF, gluteofemoral depot; Abd, abdominal depot.
correlations between miR196b and HOXA7, HOXA10, and HOXA11, three HOX genes located adjacent to each other on chromosome 7 (Table 2 and Figure 4A). miR196a2 gene expression was negatively correlated with HOXD8. miR196a1 expression was correlated with HOXB7, HOXB8, and HOTAIR (HOX antisense intergenic RNA), a well-studied long noncoding RNA previously identified as a regulator of adipogenesis by our labs (26). Finally, miR204 expression was positively correlated with HOXC10 expression (Table 2 and Figure 4B).

**miR196 decreases HOX gene expression in human preadipocytes**

To determine whether miR196a can also influence the expression of mRNA in human adipose tissue, we transfected human abdominal preadipocytes with the corresponding miRNA mimics, as described in Methods. The list of the highest-scoring putative gene targets as predicted by TargetScan (http://www.targetscan.org/) for the miR196 family is shown in Supporting Information Table S1. This analysis predicted that some HOX genes are putative targets of miR196.
including HOXA5, HOXA9, HOXB8, and HOXC8, all of which have been shown to be upregulated in the abdominal depot in both sexes, whereas HOXB7 has been shown to be upregulated in the abdominal depot only in males (13). Cell transfection resulted in an increase of miR196 expression between 100- and 900-fold and a reduction of 3, 3.5, 3.4, and 5 times of HOXA7, HOXB7, HOXC8, and HOXB8 expression, respectively. Expression of other HOX genes (such as HOXA5 and HOXA9) or HOTAIR was not

![Figure 4](https://www.obesityjournal.org)

**Figure 4 Relationships between selected microRNAs and HOX gene expression.** The relationships of the differentially expressed HOX genes, miR196a, miR196b, and miR204, were determined using SEM (see Methods for details) to build a coexpression network. HOX genes are represented by numbered boxes (not to scale). Genes upregulated in the gluteal depot as compared to abdominal are in green; genes downregulated in the gluteal depot are in red. Arrows indicate a positive correlation; dashed arrows indicate negative correlation. (A) miR196a2 and miR196b showed interactions with HOX A cluster on chromosome 7. (B) miR196a1/a2 and miR204 showed interactions with HOX B cluster on chromosome 17, HOX C cluster on chromosome 12, and HOX D cluster on chromosome 2. (C) Expression of hOXA7, HOXB7,HOXB8, and HOXC8 in miR196a transfected abdominal preadipocytes. Bar graph represents mean and SEM of relative expression of miR196a at 72h post transfection. HOX gene relative expression was plotted for each participant. Circles represent cells transfected by hsa-Control, and squares represent cells transfected by hsa-miR196a. Gene expression was normalized to Cyclophilin A. *Paired t test P < 0.01. NTC, negative control.

**TABLE 2 Correlations between miRNA expression and HOX gene expression**

|       | HOXA7  | HOXA9  | HOXA10 | HOXA11 | HOXB7 | HOXB8 | HOXC9  | HOXC10 | HOXD8 |
|-------|--------|--------|--------|--------|--------|--------|--------|--------|-------|
| miR196a1 |       | $eta = -0.7404$ |       |       |       |       |       |       |       |
|        |       | $P < 0.01$  |       |       |       |       |       |       |       |
| miR196a2 |       |       |       |       |       |       |       | $eta = -0.5221$ |       |
|        |       |       |       |       |       |       |       | $P < 0.05$  |       |
| miR196b | $eta = 0.5013$ |       |       |       |       |       |       |       |       |
|        | $P < 0.05$  |       |       |       |       |       |       |       |       |
| miR204 |       |       |       |       |       |       |       |       | $eta = 0.5757$ |
|        |       |       |       |       |       |       |       |       | $P < 0.05$  |
influenced by miR196 modulation (data not shown). Interestingly, we did not observe a modification of gene expression when we modulated miR196a expression in the corresponding GF preadipocytes where expression was already high (data not shown).

Discussion

Since their discovery, miRNAs have attracted considerable interest, and the volume of research is growing exponentially. Their importance in adipose tissue formation came first from the observation that inhibition of Drosha and Dicer, critical miRNA-processing enzymes, in human mesenchymal stem cells inhibited the differentiation of these cells into adipocytes (27). Since then, several global profiling studies have been performed to identify new miRNA candidates implicated in adipose tissue development and biology (28-30).

In the present study, we hypothesized that miRNAs might be differentially expressed in abdominal and GF adipose tissue and that they could play a role in adipose tissue distribution. Rantalainen et al. analyzed global miRNA expression in GF and abdominal adipose tissue in human subjects using an expression quantitative trait loci method (19). Among all miRNAs identified with a higher expression in GF adipose tissue, three are in common with our results: miR196a, miR196b, and miR204. Our actual method using next-generation sequencing presents an advantage, as it detects miRNAs from a library of more than 1,400 miRNAs instead of detecting defined miRNAs. We observed for the first time a specific upregulation of both isoforms miR196a1 and miR196a2, together with miR205 in the GF depot. To identify miRNA targets, we combined our miRNA data with previous mRNA data (generated using the same subjects) and performed in silico analysis (IPA and SEM). IPA revealed the central position of beta-estradiol, hormones known to influence fat distribution, in the first gene network (including miR203, miR204, and miR205), suggesting a regulation of adipose tissue miRNA expressions by sex hormones. The effect of these molecules on miR205 and other miRNAs merits further investigation. Unfortunately, in this study, the low number of subjects did not allow us to perform sex-specific analysis, and in order to limit the influence of sex hormones, we decided to focus on miRNAs upregulated in GF in both sexes.

Previous studies have shown interaction of some miRNAs and transcription factors influencing adipose tissue biology (31,32). Our current bioinformatics analyses showed potential interaction between miR196a/b and other molecules such as KRT5, ANXA1, or GFI1, but none known to be differentially regulated in human adipose tissue. We then focused on the potential interaction between miRNAs and HOX genes that we had recently identified as potential contributors to functional and morphological differences between lower-body (GF) and upper-body (abdominal) subcutaneous adipose tissues (13,33). Interestingly, we established an interaction between the miR196 gene family and HOX gene expression in accordance with their genomic location (34,35). In silico analysis predicts that miRNAs identified in the same chromosomal region as HOX clusters preferentially target loci (in cis) HOX miRNAs. Our correlation data, pathway analysis, and then our in vitro experiments suggested these interactions in adipocytes. Further experiments, such as knockdown of miR196 genes, will be necessary to identify their actual targets in adipocytes.

We observed that a higher expression of miR196a in the GF depot led to a decrease of fat development in this depot (characterized by a lower hip circumference; Figure 3C). In addition, a higher expression of miR196a in the abdominal depot seems beneficial because it is associated with a decrease of fat mass. We also showed that miR196a overexpression is associated with a decreased expression of HOX8C (Figure 4C), a gene known to be involved in the development of brown adipose tissue, targeted tissue to combat obesity and fat development. Our data suggest that a repression of miR196a expression in the GF depot would be a good therapeutic target to stimulate the expansion of the GF depot. This hypothesis merits further exploration.

The present study did not find evidence for cell-autonomous expression of all miRNAs differentially expressed in adipose tissue, suggesting that the microenvironment or the hormonal milieu in vivo may be important. Alternately, epigenetic marks may be erased in the transition to an artificial cell culture system. For example, hypoxia or inflammatory cytokines, both known to be increased in subjects with obesity, could influence miRNA expression in adipose tissues (36,37).

Adipose tissue is a heterogeneous tissue in which miRNAs could be found in all cell types, including adipocytes, resident inflammatory cells, preadipocytes, vascular smooth muscle cells, endothelial cells, and pericytes. Moreover, the proportion of each type of cell varies depending on obesity or diabetes status. In the future, it will be crucial to determine which cells express each miRNA to establish their role in adipose tissue biology.

In conclusion, the miR196 gene family is upregulated in the GF depot compared to the abdominal depot, independent of gender. Bioinformatics analysis identified a complex system of interaction of miR196 and HOX genes, which are also found in vitro in human preadipocytes isolated from abdominal adipose tissue, in which miR196a overexpression decreased HOX gene expression. Further in vitro experiments are necessary to determine a direct or indirect effect.

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