Objective: Peripheral lower body fat is associated with lower cardiometabolic risk. Physiological differences in gluteal compared with abdominal subcutaneous (sc) adipocyte functions are known but the molecular basis for depot differences in adipocyte function is poorly understood. Our goal is to identify novel gene regulatory pathways that underlie the heterogeneity of human fat distribution.

Methods: Abdominal and gluteal adipose tissue aspirates obtained from 35 subjects (age = 30 ± 1.6 years; BMI = 27.3 ± 1.3 kg/m²) were analyzed using Illumina microarrays and confirmed by RT-PCR. The HOTAIR gene was stably transfected into primary cultured human abdominal sc preadipocytes using a lentivirus and effects on adipogenic differentiation were analyzed.

Results: A long noncoding RNA, HOTAIR that was expressed in gluteal but not in Abd sc adipose tissue was identified. This difference was retained throughout in vitro differentiation and was maximal at day 4. Ectopic expression of HOTAIR in abdominal preadipocytes produced an increase in differentiation as reflected by a higher percentage of differentiated cells, and increased expression of key adipogenic genes including PPARγ and LPL.

Conclusions: HOTAIR is expressed in gluteal adipose and may regulate key processes in adipocyte differentiation. The role of this IncRNA in determining the metabolic properties of gluteal compared with abdominal adipocytes merits further study.

Introduction

Whereas the mass of abdominal subcutaneous (sc) adipose tissue is associated with increased risk for metabolic disease, the amount of lower body sc (reflected in hip circumference) is independently associated with a protective effect on risk for myocardial infarction and type 2 diabetes (1-3). The exact physiological, metabolic, or endocrine mechanism(s) underlying these beneficial effects are not known.

Our recent studies profiling gene expression in these two depots identified major differences in the expression of HOX genes, well-

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**Abbreviations**

Abd, Abdominal; FABP4, Fatty Acid Binding Protein 4; Glut, Glucose; HOTAIR, Homeobox Transcript Antisense Intergenic RNA; HOX, Homeobox; lncRNA, long non-coding RNA; LPL, Lipoprotein Lipase; miRNA, MicroRNA; PCR2, Polycomb Repressive Complex 2; PPARγ, Peroxisome Proliferator-Activated receptor γ; scWAT, subcutaneous White Adipose Tissue; WHR, Waist to Hip Ratio

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Intriguingly, HOXC13, a gene shown in a genome-wide association study to be associated with fat distribution (5) especially in females, was expressed only in the gluteal depot. These differences in developmental gene expression were cell autonomous in cultured preadipocytes and maintained after differentiation (4).

Although traditionally, the regulation of gene expression in adipocytes has focused on nuclear transcription factors as key regulators of adipogenesis and lipogenesis (6), the role of noncoding RNAs, including miRNAs and long non-coding RNAs (lncRNA) is increasingly recognized. The lncRNAs are unique in that they regulate gene transcription in a fashion similar to transcription factors in the nucleus (7). Mechanistically, they can bind directly to DNA or bridge transcription factors onto the nucleus and exert powerful effects on gene transcription (8).

Re-examining our microarray results (4), we noted that the lncRNA HOTAIR was expressed in Glu but not Abd adipose tissue. HOTAIR is one of the few lncRNA with well described molecular function (9). Located within the human HOXC cluster, HOTAIR downregulates the HOXD cluster genes in trans via the recruitment of PRC2, a silencing complex involved in histone methylation. Overexpression of HOTAIR in several types of human cancers has been linked to metastasis and cancer progression (10).

In this report, we used qPCR to confirm that HOTAIR is expressed only in gluteal adipose tissue, examined its expression in adipocyte and stromal cell fractions, and assessed the effect of ectopic expression of HOTAIR in differentiation of Abd preadipocytes, where its expression is essentially absent.

**Methods**

The method of recruitment, clinical and biochemical parameters of subjects and some of the microarray methods are presented in Kastergiou K et al. (4). Briefly, paired abdominal and gluteal scWAT samples were obtained from 21 men and 14 women (age = 30 ± 1.6 years; BMI = 27.3 ± 1.3 kg/m²; WHR = 0.87 ± 0.02) and total RNA was isolated with QIAGEN spin columns and analyzed using the Sentrix Human-6 V2 Expression BeadChip (Illumina, San Diego, CA). Clinical Trial Registration Number: NCT00704197

**Real time quantitative RT-PCR**

Gene expression was assessed by real-time PCR using a ViiA7 sequence detection system (Life technologies) and Taqman technology suitable for relative gene expression quantification using the following parameters: one cycle of 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 1 min (Table 1).

**Isolation of adipose fractions and in vitro experiments**

For these studies, we used adipose tissue biopsies obtained from 4 healthy volunteers (3 M, 1 F, age 28.3 ± 4.4 y, BMI 26.4 ± 3 kg/m²) obtained at Boston Medical Center, after approval by the IRB and providing written informed consent. Stromal-vascular fractions were isolated by collagenase digestion of abdominal and gluteal sc adipose tissues. They were plated, grown, and differentiated as previously described (11). Cells were harvested across differentiation (d 0-14), RNA was extracted, and target genes were measured as described above. Paired samples of adipose tissue, isolated adipocyte and stromal fractions were also flash frozen in liquid nitrogen and RNA extracted (4).

**Transfection of preadipocytes**

HOTAIR lentivirus was produced by Capital biosciences (Rockville, MD). It was generated by cotransfection of 293T packaging cells with pLV-CMV-HOTAIR-mKate2-2A-Puro plasmid and Packing Mix. HOTAIR expression is under the CMV promoter, coexpression of Red Fluorescence Protein, mKate2 protein, and Puromycin resistance marker is driven by SV40 promoter. HOTAIR and control lentiviruses were transduced into preadipocytes overnight at MOI = 10 in the presence of polybrene (8 µg/ml). Five days later transduced cells were selected with puromycin (1 µg/ml for one week). Overexpression was made twice in two independent cells. Cells were then differentiated according to the protocol described in (11).

**Western blot analysis**

Cells were harvested in cell lysis buffer (Cell Signaling) with 5% SDS and protease inhibitors (Pierce); 5-10 µg total protein was resolved in 10% Tris-SCC gels (Biorad) and transferred to PVDF membranes. Membranes were probed for FABP4 (a gift from Dr. Judith Storch at Rutgers University) and adiponectin (BD Biosciences), Chemiluminescence images were captured using an Imager (LAS 4000, Fuji).

**Results**

HOTAIR is expressed only in the gluteal depot

We identified HOTAIR as a long noncoding RNA expressed in gluteal but not abdominal sc adipose tissue in both sexes (Figure 1A). These microarray results were verified using qPCR in the same group of subjects: HOTAIR was at the detection limit in abdominal sc adipose tissue samples (CT 37-40) and expressed in all gluteal adipose tissue samples (CT 29-36, Figure 1B). HOTAIR gene expression was similar in males and females. HOTAIR expression was highly variable and enriched >10-fold in isolated gluteal adipocytes compared with gluteal adipose tissue, but was also expressed in gluteal stromal cells [tissue levels: 4.5 ± 4.1 arbitrary units (AU), adipocytes: 58.8 ± 23.7, stromal-vascular fraction 13.5 ± 10.5 AU]. In contrast, HOTAIR was at the limit of detection in both cell fractions of abdominal adipose tissue. To determine whether gluteal adipocytes expressed HOTAIR in a cell autonomous, depot-specific

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**TABLE 1 List of primer/probes commercially available from Life Technologies (Single Tube TaqMan® Gene Expression Assays) used for real-time PCR**

| Gene      | Exon boundary | Amplicon length |
|-----------|---------------|-----------------|
| HOTAIR    | 1-2 and 2-3   | 152             |
| HOXD3     | 2-3           | 70              |
| HOXD4     | 1-2           | 91              |
| Leptin    | 6-7 and 5-6   | 111             |
| Adiponectin | 3-4 and 2-3   | 71              |
| PPARγ     | 4-5 and 5-6   | 90              |
| LPL        | 6-7 and 7-8   | 103             |
| GAPDH     | 6-7           | 93              |

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manner, we examined expression in primary cultures of preadipocytes from both depots. The depot difference in HOTAIR gene expression was maintained ex vivo throughout differentiation. The level of expression of HOTAIR achieved with OE in abd sc adipocytes was substantially higher than endogenous levels observed in gluteal preadipocytes (over 1000-fold). As shown in Figure 2B HOTAIR overexpression enhanced differentiation into adipocytes, reflected by a greater percentage of cells differentiated (Figure 2B) and higher expression of late markers of mature adipocytes, adiponectin, and FABP4 (Figure 2C), as well a 5.6-fold increase of PPARγ gene expression, key transcriptional factor of adipogenesis and a 3.7-fold increase of LPL gene expression, main enzyme of lipid storage in adipocytes (Figure 2D). Western blot analysis showed a parallel increase in adiponectin and FABP4 protein levels at D14 of the differentiation (Figure 2C). Transfected cells unexpectedly showed a small increase in HOXD4 and HOXD3 gene expression. HOTAIR overexpression had no effect on preadipocyte proliferation rate (data not shown).

**Discussion**

Although depot differences in human adipocyte morphology and metabolic function, even among sc depots, are well established, the physiologic, cellular, and molecular underpinnings of variations in regional adipose tissue biology are poorly understood. Here, we establish that the lncRNA HOTAIR was expressed in gluteal adipose tissue, with similar levels in men and women. HOTAIR was expressed in both adipocyte and stromal cell fractions, and was enriched in the adipocyte fraction suggesting potential functions in differentiation and mature adipocyte function. Moreover, HOTAIR was expressed in a cell autonomous manner by gluteal preadipocytes, and preliminary evidence is consistent with a role in the regulation of adipogenesis.

Noncoding RNAs are known to be involved in developmental processes. HOTAIR is able to operate not only as a “guide” lncRNA, but also as a “scaffold” that bind and bring together two protein complexes that cooperate to establish a repressive chromatin state. Although several studies document the importance of specific miRNAs in adipogenesis some data are available on lncRNAs. Sun et al. has documented lncRNAs that are regulated during white (3T3-L1) and brown adipogenesis (12). HOTAIR is known to regulate the transcriptional silencing of genes in the HOXD locus (9). Surprisingly we observed a small increase of HOXD3 and HOXD4 expression after HOTAIR transfection. However, these HOX D genes are not completely silenced in human adipose tissue. It will be important to conduct additional studies of HOTAIR targets at more physiologically relevant levels of HOTAIR overexpression, and to assess the effect of its knockdown in gluteal-derived preadipocytes and adipocytes.

Associative data support the role of circulating estradiol in maintaining gynoid body fat distribution (13) and estrogen is known to increase HOTAIR gene expression in other cells types (14). Nevertheless, similar levels of HOTAIR were detected in gluteal adipose tissue from males and females, and our in vitro experiments were performed in absence of added estrogen or other sex hormones. Thus, there is a cell-autonomous expression of HOTAIR in gluteal

**Ectopic overexpression of HOTAIR enhances the differentiation of abdominal preadipocytes**

To evaluate potential functional roles of HOTAIR in adipose tissue, we overexpressed the HOTAIR gene with lentivirus in two independent stable cultures of abdominal sc preadipocytes and differentiated them into adipocytes using a standard protocol (11). Fluorescent microscopy (Figure 2A) confirmed that HOTAIR construct was uniformly expressed in all cells prior to and after differentiation. The level of expression of HOTAIR achieved with OE in abd sc adipocytes was substantially higher than endogenous levels observed in gluteal preadipocytes (over 1000-fold). As shown in Figure 2B HOTAIR overexpression enhanced differentiation into adipocytes, reflected by a greater percentage of cells differentiated (Figure 2B) and higher expression of late markers of mature adipocytes, adiponectin, and FABP4 (Figure 2C), as well a 5.6-fold increase of PPARγ gene expression, key transcriptional factor of adipogenesis and a 3.7-fold increase of LPL gene expression, main enzyme of lipid storage in adipocytes (Figure 2D). Western blot analysis showed a parallel increase in adiponectin and FABP4 protein levels at D14 of the differentiation (Figure 2C). Transfected cells unexpectedly showed a small increase in HOXD4 and HOXD3 gene expression. HOTAIR overexpression had no effect on preadipocyte proliferation rate (data not shown).
FIGURE 2 Effects of HOTAIR overexpression in abdominal sc preadipocytes. Abd preadipocytes were stably transfected with a control or a pLV-CMV-HOTAIR-mKate2-2A-Puro lentivirus where HOTAIR is under the CMV promoter and mKate2 protein, Puromycin resistance marker and red fluorescent protein are under SV40 promoter. Overexpression of HOTAIR was confirmed by fluorescence microscopy at Day 0 (preadipocyte stage) and Day 14 (adipocyte stage) of the differentiation (A). qPCR analysis showed a 2500-fold increase of HOTAIR gene expression throughout differentiation (A). Brightfield pictures taken at the end of the differentiation showed that the number of differentiated preadipocytes increased in transfected cells (HOTAIR) compared with control cells, (representative picture of n = 4 experiments; B). Sequential increase of gene expression of adiponectin (ADPN) and FABP4 was higher in transfected cells compared with control cells (CTRL; n = 2; C). Western blot analysis at the end of the differentiation confirmed a higher level of Adiponectin and FABP4 proteins in transfected cells (representative western blot of n = 2; C). HOTAIR transfection leads to an increase of expression of late adipocyte marker genes (Leptin, PPARγ, and LPL) and HOXD3 and HOXD4 genes (D). qPCR analysis was performed at the end of the differentiation (D14) and compared with nontransfected cells. Lines connect results from two independent set of stable control and HOTAIR cell cultures. Ctrl: Control cells; OE: Overexpressed cells.
adipose tissue. In conclusion, we show HOTAIR is expressed in gluteal adipose tissue, mainly in the adipocyte fraction, and in cultures of preadipocytes. A large increase in HOTAIR expression can drive an increase in the differentiation in abdominal preadipocytes, which express undetectable levels of HOTAIR. These results provide impetus for detailed studies of the studies gene targets of HOTAIR and other lncRNAs in determining regional adiposity.

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