Evidence for a role of developmental genes in the origin of obesity and body fat distribution

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Obesity, especially central obesity, is a hereditary trait associated with a high risk for development of diabetes and metabolic disorders. Combined gene expression analysis of adipocyte- and preadipocyte-containing fractions from intraabdominal and subcutaneous adipose tissue of mice revealed coordinated depot-specific differences in expression of multiple genes involved in embryonic development and pattern specification. These differences were intrinsic and persisted during in vitro culture and differentiation. Similar depot-specific differences in expression of developmental genes were observed in human subcutaneous versus visceral adipose tissue. Furthermore, in humans, several genes exhibited changes in expression that correlated closely with body mass index and/or waist/hip ratio. Together, these data suggest that genetically programmed developmental differences in adipocytes and their precursors in different regions of the body play an important role in obesity, body fat distribution, and potential functional differences between internal and subcutaneous adipose tissue.

In the present study, we have explored the hypothesis that patterns of fat distribution and, perhaps, to some degree, obesity itself may have a developmental genetic origin. Indeed, we find major differences in expression of multiple genes involved in embryonic development and pattern specification between adipocytes taken from intraabdominal and s.c. depots in rodents and humans. We also demonstrate similar differences in the stromovascular fraction (SVF)-containing preadipocytes and that these differences persist in culture. Most importantly, we demonstrate that some of these developmental genes exhibit changes in expression that are closely correlated with the level of obesity and the pattern of fat distribution.

Results

Genes Expression Differences Between Intraabdominal and s.c. Adipose Tissue of Mice. Several studies have reported differences in gene expression (19–22) and proliferative capacity (23–28) between fat taken from different depots in rodents and humans, suggesting that genetic programming could affect specific adipose depot development. To address this hypothesis, we performed gene expression analysis of both adipocytes and SVF containing preadipocytes taken from s.c. (flank) fat and intraabdominal (epididymal) fat, using Affymetrix U74Av2 microarrays with 8,017 probe sets representing 6,174 different annotated genes (Fig. 1). Of these, 197 genes were found to have joint differential expression in both cell fractions between the two tissue beds by using stringent statistical criteria with a two-tailed test value for both cell fractions <0.05 and a positive false discovery rate <0.05 (see Supporting Methods and Table 2, which are published as supporting information on the PNAS web site). This list was assessed against an a priori set of 198 annotated genes involved in embryonic development and pattern specification on the array (see Supporting Methods and Table 3, which are published as supporting information on the PNAS web site). Twelve of these developmental genes were found among the differentially expressed genes, representing a 1.9-fold enrichment (P = 0.006) compared with the 6,174 annotated genes on the array (Table 1).

Among these 12 genes, seven genes had higher levels of expression in intraabdominal epididymal SVF and/or adipocytes (Nrf21, Thbd, HoxA5, HoxC8, Gpc4, Hrmt12, and Vdr) and five genes had higher levels of expression in s.c. SVF and/or adipocytes (Tbx15, Sho2, En1, Sfp2, and HoxC9). Of the seven genes from intraabdominal group, we decided to focus our analysis on the five most significant genes, including two homeo box genes, HoxA5 and HoxC8; Nrf21, nuclear receptor subfamily 2 group F member 1, also known as COUP-TFI, an orphan member of the steroid receptor superfamily thought to be involved in organogenesis (29); glypican 4 (Gpc4), a cell-surface heparan sulfate proteoglycan involved in

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Abbreviations: SVF, stromovascular fraction; WHR, waist/hip ratio; BMI, body mass index; Thbd, thrombomodulin; gPCR, quantitative PCR.

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cell division and growth regulation (30); and thrombomodulin (Tibd), a surface glycoprotein of endothelial and placental cells (31). We also studied all five genes from the s.c. group of genes including the homeobox gene HoxC9; short stature homeobox 2 (Shox2) a transcription factor with homeodomain expressed during embryonic development (32); Tbox15 (Tbx15), a transcription factor involved in craniofacial and limb development in the mouse (33); engrailed 1 (En1), the mouse homologue of a Drosophila patterning gene (34); and secreted frizzled-related protein 2 (Sfrp2), a soluble modulator of Wnt signaling (35).

Confirmation of Interdepot Gene Expression Differences by Quantitative PCR (qPCR). These differences of expression in genes involved in embryonic development and pattern specification were confirmed by qPCR. In whole tissue, all predominantly s.c. genes Tbx15, Shox2, En1, Sfrp2, and HoxC9 were more highly expressed in s.c. adipose tissue than in intraabdominal (epidymidal) fat, with the most marked differences observed for Tbx15, Shox2, and En1 expression (39-, 23-, and 5.4-fold, respectively; P = 0.005, 0.018, and 0.008, respectively) (Fig. 2). Conversely, all predominant intraabdominal genes, Nr2f1, Gpc4, Thbd, HoxA5, and HoxC8, were significantly more expressed in intraabdominal adipose tissue than in s.c. adipose tissue by 2.1- to 3.5-fold (all P = 0.05) (Fig. 3).

Likewise, differences were confirmed in isolated adipocytes and stromavascular cells obtained from both depots by qPCR. Thus, both adipocytes and SVF cells isolated from s.c. adipose tissue expressed higher levels of all s.c. genes, Tbx15 [140- and 460-fold (P = 0.001 and 0.013)], Shox2 [20- and 205-fold (P = 0.006 and 0.012)], En1 [12.3- and 4.9-fold (P = 0.0006 and 0.0007)], Sfrp2 [2.6- and 4.5-fold (P = 0.001 and 0.04)], and HoxC9 [1.8- and 2.1-fold (P = 0.023 and 0.06)] (Fig. 2B). Conversely, adipocytes and SVF from epididymal adipose tissue expressed higher levels of intraabdominal genes Nr2f1, Gpc4, Thbd, HoxA5, and HoxC8 [5.4- and 7.8-fold (P = 0.006 and 0.003)], 2.1- and 1.5-fold (P = 0.003 and 0.05), 3.8- and 0.7-fold (P = 0.004 and 0.3), 1.6- and 2.2-fold (P = 0.04 and 0.02), and 3.8- and 1.7-fold (P = 0.009 and 0.02), respectively] (Fig. 3B).

Table 1. Developmental and patterning genes showing differential expression in adipocytes and SVF of adipose tissue

<table>
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<tr>
<th>Gene ID</th>
<th>Probe set no.</th>
<th>Ae, Asc vs. Se, Ssc</th>
<th>P value,</th>
<th>Gene title</th>
<th>Gene symbol</th>
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<tr>
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Ae, epididymal isolated adipocytes; Asc, s.c. isolated adipocytes; Se, epidydimal SVF; Ssc, s.c. SVF; pFDR, positive false discovery rate. Bold represents the tissue bed with the highest level of expression.
Interdepot Differences in Gene Expression Are Independent of Extrinsic Factors. To determine whether these differences in gene expression were cell-autonomous, preadipocytes (SVF) taken from intraabdominal (epididymal) or s.c. adipose were placed in culture in defined serum-free medium and subjected to in vitro differentiation. After 6 days, all of the predominantly s.c. genes and all of the predominantly epididymal genes maintained their interdepot differences of expression (Figs. 2C and 3C). Thus, differences of developmental gene expression between depots are independent of extrinsic factors, such as innervation, blood flow, the level of oxygenation and nutrients, or any other interstitial factors.

Interdepot Differences of Expression in Humans. Because the striking interdepot differences for expression of these developmental genes between s.c. and intraabdominal fat in mice appeared to be intrinsic and present in both the preadipocyte and adipocyte fractions, we decided to determine whether similar differences might be present in human adipose tissue. To address this question, 53 lean subjects (22 males and 31 females with BMI <25) with normal fat distribution (WHR for males, 0.80–1.06; WHR for females, 0.62–0.87) were subjected to abdominal s.c. and visceral adipose tissue biopsies, and gene expression for the human homologues of each of these developmental genes was assessed by using qPCR.

As observed in mice, Nr2f1, Thbd, HoxA5, and HoxC8, which showed higher expression in epididymal fat, showed a higher level of expression in visceral adipose tissue of humans in both males and females (Fig. 4). In addition, for these genes the magnitude of interdepot differential gene expression in humans was even greater than that in mice (Nr2f1, 461- and 894-fold; Thbd, 124- and 147-fold; HoxA5, 23- and 24-fold; HoxC8, 1,210- and 1,100-fold, for males and females, respectively). Glypican 4 (Gpc4) expression in humans also showed a strong differential expression; however, in lean humans this gene was more highly expressed in s.c. than in visceral adipose tissue, with a 5.4-fold difference in males and a 4.8-fold difference in females.

The group of s.c. genes also showed significant and differential patterns of expression between depots in humans. In this case, two of the genes, Shox2 and En1, presented a pattern of expression in humans in the same direction as in mice, and in the case of En1 the
Differential expression was of extreme magnitude (17,500- and 42,500-fold for males and females, respectively) (Fig. 4). As in mice, HoxC9 expression was found significantly higher in s.c. than in visceral adipose tissue (2.3-fold); however, in humans this difference was gender-specific and was not present in males. Tbx15 and Sfrp2 also showed markedly different expression in humans; however, in humans these genes were more highly expressed in visceral adipose tissue than in s.c. adipose tissue in both genders (Tbx15, 27.1-fold in males and 38.7-fold in females; Sfrp2, 950-fold in males and 1,200-fold in females) (Table 4, which is published as supporting information on the PNAS web site). Three of the 10 developmental genes showed significant relationships to BMI or WHR. HoxA5 expression in both visceral and s.c. adipose tissue significantly increased with BMI in males ($R = 0.448, P < 0.0001$; $R = 0.292, P = 0.0034$, respectively) and females ($R = 0.535, P < 0.0001$; $R = 0.361, P = 0.0002$, respectively) (Fig. 5A). This correlation was more marked in visceral than in s.c. adipose tissue in both genders. In addition, there was a significant positive correlation of HoxA5 expression with WHR in visceral and s.c. adipose tissue for both males ($R = 0.446, P < 0.0001$; $R = 0.479, P < 0.0001$, respectively) and females ($R = 0.580, P < 0.0001$; $R = 0.449, P < 0.0001$, respectively) (Fig. 5B).

In human adipose, there were very strong correlations of Gpc4 expression with BMI and WHR in both males and females. In this case, the correlation in the two depots was in opposite directions, with decreasing Gpc4 expression in s.c. adipose tissue with increasing BMI (males, $R = 0.74, P < 0.0001$; females, $R = 0.735, P < 0.0001$) and WHR (males, $R = 0.575, P < 0.0001$; females, $R = 0.730, P < 0.0001$) and increasing Gpc4 expression in visceral adipose tissue with increasing BMI (males, $R = 0.525, P < 0.0001$; females, $R = 0.507, P < 0.0001$) and WHR (males, $R = 0.596, P < 0.0001$; females, $R = 0.5, P < 0.0001$) (Fig. 5A). In addition, the shape of the relationship was different, being fairly linear in visceral adipose tissue, whereas in s.c. adipose tissue Gpc4 expression decreased abruptly as individuals went from normal BMI (20–25) to overweight (BMI > 25) or obese (BMI > 30) levels. Likewise, in s.c. adipose tissue Gpc4 expression displayed a curvilinear negative correlation with very low levels in males with WHR > 1.1 and females with WHR > 0.95.

The most profound correlations with BMI and WHR were observed for Tbx15 expression in visceral adipose tissue. As with Gpc4, there was a strong exponential negative relationship with a marked decrease in Tbx15 expression as BMI progressed from normal to overweight or obese levels. This was true in both males ($R = 0.706, P < 0.0001$) and females ($R = 0.852, P < 0.0001$) (Fig. 5A). There was also a strong exponential negative relationship between Tbx15 expression and WHR in visceral adipose tissue with marked declines above WHR of 1.05 for males ($R = 0.604, P < 0.0001$) and 0.95 for females ($R = 0.817, P < 0.0001$) (Fig. 5B). By contrast, Tbx15 expression showed a more modest positive correlation with both BMI and WHR in s.c. adipose tissue of both males ($R = 0.282, P = 0.00047$; $R = 0.406, P < 0.0001$) and females ($R = 0.191, P = 0.0587; R = 0.345, P = 0.0005$). However, in all cases, expression of Tbx15 in s.c. tissue was much lower than the level of expression in visceral adipose tissue of lean individuals. Thus, HoxA5, Gpc4, and Tbx15 expression in adipose tissue were strongly correlated with the level of obesity, as well as adipose tissue distribution, especially Tbx15 expression in visceral fat.

**Discussion**

Obesity is a multifactorial disorder influenced by a mixture of genetic and environmental factors, including control of appetite and energy expenditure, availability and nutritional content of food, and development of adipocyte cell mass. Furthermore, obesity occurs with different degrees of fat accumulation in different depots, and these are associated with different metabolic consequences with intraabdominal (visceral) accumulation of fat producing a much greater risk of diabetes, dyslipidemia, and accelerated atherosclerosis than s.c. (peripheral) fat.

Although obesity and body fat distribution are clearly hereditary traits, the role of developmental genes in obesity and fat distribution has received surprisingly little attention. Previous work has shown that SVF taken from different adipose depots (23–28) and from obese versus lean individuals show different propensities to differentiate when placed in tissue culture in vitro (36). In addition, the rate of lipolysis in adipose tissue taken from s.c. sites is lower than that of adipose tissue from visceral or omental sites (37). Furthermore, the lipolytic effect of catecholamines is weaker and the
antilipolytic effect of insulin is more pronounced in s.c. than in visceral adipose tissue (38, 39).

Characterization of differences in gene expression between human s.c. and visceral adipose tissue also suggest genetic/developmental heterogeneity. Acylation-stimulating protein and angiotensinogen mRNA levels are higher in visceral adipose, whereas the levels of leptin, peroxisome proliferator-activated receptor γ, glucose transporter 4, glycogen synthase, and cholesterol ester transfer protein are higher in the s.c. depot (40, 41). In a survey of genes differentially expressed in s.c. and visceral adipose tissue in men, Vohl et al. (21) also noted differences in genes involved in lipolysis, cytokine secretion, Wnt signaling, C/EBPα, and some HOX genes. We also observed differences in large and small adipocytes taken from normal and fat insulin receptor knockout mice with regard to function and gene and protein expression (42–44). In the present study, therefore, we explored the hypothesis that developmental genes might play an important role in obesity and body fat distribution in both rodents and humans.

Using microarray and qPCR analysis, we demonstrated that 197 genes are differentially expressed in both adipocytes and SVF containing preadipocytes from s.c. and intraabdominal depots of the mouse and that at least 12 are genes known to play a role in early development and pattern specification. Of these, Tbx15, Shox2, En1, Sfrp2, and Hox9 were more highly expressed in cells of s.c. adipose tissue, whereas Nr2f1, Gpc4, Thbd, HoxA5, and HoxC8 were more expressed in intraabdominal adipose tissue. These differences in gene expression are intrinsic and persist during in vitro culture and differentiation, indicating that they are cell-autonomous and independent of tissue microenvironment. Because the expression of these developmental genes emerges during embryogenesis, before any white adipose tissue can be detected, and is maintained during adult life, this would suggest that different adipocyte precursors are responsible for a specific adipose depot development and may participate later in the functional differences observed between internal and s.c. adipose depots.

Although all of the genes that were differential in rodents were also differential in humans, in some cases the direction of difference was different in the two species. This difference of direction may reflect the fact that fat was not taken from identical depots in the two species or may simply represent differences between development in these two species. Other differences in gene expression have also been observed between humans and rodents. Thus, leptin exhibits a higher expression in s.c. than omental adipose in humans (40, 41), whereas, in mice, leptin expression is higher in intraabdominal (epididymal) fat than s.c. fat (45). Likewise, the differential expression of α2-adrenergic receptor expression observed in humans (higher in s.c. adipose than in omental) (38) is not observed at all in mice, which do not express α2-adrenergic receptors in adipose tissue (46). Conversely, β3-adrenergic receptors are widely expressed in mouse adipose tissue, whereas little or no expression has been reported in human adipose (47). In our case, the inter-depot differences of expression for developmental genes Shox2, En1, Nr2f1, HoxA5, HoxC8, and Thbd were preserved from mice to humans independent of gender, whereas inter-depot differential expression of Hox9 in humans occurred only in females, and Tbx15, Sfrp2, and Gpc4 exhibited opposite directions of differential expression in mice and humans. In both species, what is clear is that multiple developmental genes, including those involved in anteroposterior or dorsoventral patterning, exhibit dramatic differences in the level of expression in adipose and preadipose from different regions of the body.

One of the most striking features of the expression of HoxA5, Gpc4, and Tbx15 in human adipose is not only their differential expression between depots, but also their strong correlation with BMI. This correlation is particularly true for Tbx15 in visceral fat and Gpc4 in s.c. fat, such that both genes show dramatic changes in expression as BMI goes from the normal range (BMI = 20–25) to either overweight (BMI = 25–30) or obese (BMI > 30). No other parameter related to obesity or fat mass, including serum leptin, adiponectin, or insulin, shows such a distinct change at this transition point. Indeed, if the physiological separation between lean and...
overweight/obese had not been previously defined by epidemiological criteria, one could define the overweight population by the expression level of these genes, suggesting that expression of these genes could be related to the pathogenesis of obesity.

Distribution of adipose tissue (WHR) also has a strong heritable component (12) and has been shown to better correlate with risk of diabetes and atherosclerosis than BMI (48). Increased WHR, i.e., visceral/adipose tissue appears to be linked with high WHR and by logical criteria, one could define the overweight population by the expression in s.c. adipose tissue and low levels of expression in visceral adipose tissue appear to be linked with high WHR and by extension should be correlated with higher risks for metabolic and cardiovascular complications.

Although the exact role of each of these genes in development and distribution of fat needs to be explored, the Hox, Shox, and Tbx genes are important in dorsal–ventral and anterior–posterior patterning and has shown to be correlated with higher risks for metabolic and cardiovascular complications (2–5). We find that HoxA5, Gpc4, and Tbx15 expression also with fat distribution and the expression of the latter two is an excellent marker for visceral fat accumulation. Thus, high levels of Tbx15 and Gpc4 expression in s.c. adipose tissue and low levels of expression in visceral adipose tissue appear to be linked with high WHR and by extension should be correlated with higher risks for metabolic and cardiovascular complications.

Taken together, our data suggest that genes involved in embryonic development and pattern specification in mice and humans play potentially important roles in adipocyte development and fat distribution. These results suggest that different adipocyte precursors are responsible for a specific adipose depot development in a manner similar to the scheme of differentiation defined for blood cells and other lineages (Fig. 6). These findings open an avenue of understanding fat accumulation and distribution and present therapeutic targets for this epidemic disorder.

Materials and Methods

Detailed methods are presented in Supporting Methods. In brief, adipocytes and SVF were isolated from epididymal and flank s.c. adipose tissue of C57BL/6 mice. RNA from adipose tissue, isolated adipocytes, and SVF were isolated using an RNeasy kit (Qiagen, Valencia, CA). Microarray experiments and analysis were performed as described in Fig. 1.

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