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Analysis of In Vitro Insulin-Resistance Models and Their Physiological Relevance to In Vivo Diet-Induced Adipose Insulin Resistance

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SUMMARY

Diet-induced obesity (DIO) predisposes individuals to insulin resistance, and adipose tissue has a major role in the disease. Insulin resistance can be induced in cultured adipocytes by a variety of treatments, but what aspects of the in vivo response are captured by these models remains unknown. We use global RNA sequencing to investigate changes induced by TNF-α, hypoxia, dexamethasone, high insulin, and a combination of TNF-α and hypoxia, comparing the results to changes in white adipose tissue from DIO mice. We found that different in vitro models capture distinct features of DIO adipose insulin resistance, and a combined treatment of TNF-α and hypoxia is most able to mimic the in vivo changes. Using genome-wide DNase I hypersensitivity followed by sequencing, we further examined the transcriptional regulation of TNF-α-induced insulin resistance, and we found that C/EPBβ is a potential key regulator of adipose insulin resistance.

INTRODUCTION

Obesity has become a global epidemic and predisposes individuals to insulin resistance, which in turn is a risk factor for many metabolic diseases (e.g., type 2 diabetes, hypertension, atherosclerosis, and cardiovascular diseases) and cancer (Reaven, 2005). The 3T3-L1 cell line (Green and Meuth, 1974) has been widely used to study insulin resistance in adipocytes (Knutson and Balba, 1997). Many agents are used to induce insulin resistance in differentiated 3T3-L1; these include tumor necrosis factor α (TNF-α) (Ruan et al., 2002), interleukin-1 (IL-1) (Jager et al., 2007), IL-6 (Rotter et al., 2003), free fatty acids (Nguyen et al., 2005), dexamethasone (Sakoda et al., 2000), high insulin (Thomson et al., 1997), glucosamine (Nelson et al., 2000), growth hormone (Smith et al., 1997), and hypoxia (Regazzetti et al., 2009), among others. It is unclear what features of in vivo adipose insulin resistance are captured by each of the different in vitro models and whether a combination of treatments would be able to capture the in vivo changes better than a single treatment.

In order to address these issues, we have examined the changes in transcription and transcriptional regulation induced by TNF-α, hypoxia, dexamethasone, high insulin, and a combination of TNF-α and hypoxia in differentiated 3T3-L1 adipocytes. TNF-α is a proinflammatory cytokine, which is secreted by adipocytes and macrophages in adipose tissue. Since the discovery of its role in obesity-linked insulin resistance (Hotamisligil et al., 1993), it has been widely used to induce insulin resistance in cultured cells. A more recently discovered way to induce insulin resistance is hypoxia treatment. Obese adipose tissue is hypoxic, which can lead to dysregulation of adipokine production (Hosogai et al., 2007) and insulin signaling (Regazzetti et al., 2009). Both TNF-α and hypoxia have been linked to inflammatory responses. Interestingly, dexamethasone, a synthetic glucocorticoid frequently prescribed as an anti-inflammatory agent and immunosuppressant, can also induce insulin resistance. Excessive use of dexamethasone results in Cushing’s syndrome, characterized by central obesity, insulin resistance, and other metabolic abnormalities (Andrews and Walker, 1999). Elevated endogenous glucocorticoid (e.g., the hormone cortisol in humans and corticosterone in rodents) can also lead to visceral obesity and aggravate high-fat, diet-induced insulin resistance (Masuzaki et al., 2001; Wang, 2005). Finally, high levels of insulin can induce insulin resistance, and hyperinsulinemia is postulated to be both the result and the driver of insulin resistance (Shanik et al., 2008).

To understand the relationship of these models to each other and to the in vivo setting, we have made use of high-throughput
RNA sequencing (RNA-seq) technology (Trapnell et al., 2010) and analyzed the in vitro data in parallel with adipose tissue transcriptome data from three independent diet-induced obesity (DIO) mouse models. We find that the different in vitro models show diverse transcriptional responses, each of which captures a different aspect of the in vivo data. The TNF-α and hypoxia models capture the downregulation of many glucose, lipid, and amino acid metabolic pathways observed in DIO mouse adipose tissue that are not detected in the high-insulin and dexamethasone models. Conversely, the upregulation of the inflammatory responses in DIO adipose tissue is mainly captured by the TNF-α model. Interestingly, the combination of hypoxia and TNF-α treatments resembles the actual in vivo condition more than any individual treatment.

We further explored the differences in transcriptional regulation among the in vitro models using DNase I hypersensitivity followed by massively parallel sequencing (DNase-seq), identifying many condition-specific regulatory sites. Analysis of DNase-seq data from TNF-α-induced insulin resistance revealed that in addition to NF-κB, C/EBPβ is a potential regulator of genes induced by TNF-α, and loss of PPARγ binding is likely to mediate many of the gene repression changes upon TNF-α treatment.

RESULTS

Setting Up Diverse In Vitro Insulin-Resistance Models in the 3T3-L1 Cell Line

We induced insulin-resistance models in mature 3T3-L1 cells using TNF-α, hypoxia, dexamethasone, and high insulin following established protocols (see Experimental Procedures; Figure S1). All four models exhibited compromised insulin responses as determined by phosphorylation of Akt at serine 473 (Figure 1A) and 2-deoxyglucose uptake (Figure 1B). Nevertheless, the expression of five adipocyte marker genes (Figure 1C)
varied dramatically among these models. For example, the insulin-sensitizing adipokine adiponectin (Adipoq) decreases in all models except the high-insulin model, and the insulin-sensitive glucose transporter Glut4 decreases only in the TNF-α model. The variation in these marker genes suggests that the transcriptome shifts of the four insulin-resistance models are likely to be diverse and distinct.

**Diverse Transcriptional Changes Associated with Models of Insulin Resistance**

In order to obtain a genome-wide picture of the transcriptional outcomes, we carried out RNA-seq. These data generally agreed well with the qPCR-based results for the five adipocyte marker genes (Figures 1D and 1E). The diverse effects of each method of inducing insulin resistance can be seen by analyzing glycolysis and triglyceride synthesis and degradation, key pathways of adipose metabolism. The enzymes that catalyze the irreversible steps of glycolysis, including hexokinase (Hk1 and Hk2), phosphofructokinase (Pfk1 and Pfkp), and pyruvate kinase (Pkm2), are upregulated after the hypoxia, high-insulin, and TNF-α treatments, but not the dexamethasone treatment (Figure S2A). Regarding the triglyceride synthesis and degradation pathway (Figure S2B), diacylglycerol O-acetyltransferases (Dgat1 and Dgat2), which catalyze the reaction in which diacylglycerol is covalently joined to form long-chain fatty acyl-CoAs, are repressed in the hypoxia and TNF-α models, but not the high-insulin model. Hormone-sensitive lipase (Lipe), which hydrolyzes stored triglyceride to free fatty acid, is downregulated after TNF-α treatment, as previously reported by Ruan et al. (2002); it is also repressed in the hypoxia model, but not in the high-insulin and dexamethasone models. Gene Ontology (GO) analysis (Table S1) confirms the diversity of the transcriptional responses in each condition.

**Antiadipogenesis Transcriptome Shift of DIO Mouse Adipose Tissue Captured Mainly by Treatment with TNF-α, Hypoxia, and a Combination of TNF-α and Hypoxia**

To understand how the in vitro expression changes relate to mouse insulin-resistance models, we analyzed three independent microarray data sets comparing the gene expression of adipose tissue from DIO mice and normal chow diet-fed mice (Qi et al., 2009; Fitzgibbons et al., 2011; Fujisaka et al., 2011). Although the DIO mouse expression studies used diverse conditions (Table S2), the expression changes of DIO versus control were highly correlated.

Because the in vivo data are likely to contain contributions from multiple cell types, we chose to focus our analysis on a set of genes that is most relevant to adipocytes. To this end, we identified adipogenesis-induced and adipogenesis-repressed genes that show consistent expression changes between preadipocytes and adipocytes from three independent data sets (Schupp et al., 2009; Mikkelsen et al., 2010; Sun et al., 2013) (Table S3).

The in vivo and in vitro insulin-resistance models demonstrate a striking expression pattern that is the opposite of that induced by adipogenesis (Figure 2A): many adipogenesis-induced genes are downregulated in the DIO mouse models, and conversely, many adipogenesis-repressed genes are upregulated. This anti-adipogenesis transcriptome shift is strongest in TNF-α and hypoxia 3T3-L1 models, but it can also be detected clearly in the high-insulin and dexamethasone models (Figure 2A).

Having observed that the TNF-α and hypoxia models appear to recapitulate the antiadipogenesis transcriptome shift as seen in the DIO mouse, we investigated if a combination of TNF-α and hypoxia treatments (hereafter known as cotreatment) would be better able to capture the in vivo changes. Hierarchical clustering analysis shows that the cotreatment model, like the TNF-α and hypoxia model, also exhibits the antiadipogenesis transcriptome shift (Figure 2A). Of the 640 adipogenesis-induced genes, 352 (55%) are repressed in at least one of the three DIO mouse models (Figure 2B). Conversely, 298 (44%) of the 679 adipogenesis-repressed genes are induced in at least one of the three DIO mouse models. Cotreatment with TNF-α and hypoxia recapitulates the antiadipogenesis transcriptome shift more than either the TNF-α or the hypoxia model (Figure 2B).

We went on to explore the special features of the different in vitro models. The 352 adipogenesis-induced DIO-repressed genes are highly enriched in ones encoding proteins involved in oxidation reduction (p = 6.3 × 10⁻¹¹), cell differentiation (p = 1.2 × 10⁻⁸), and various metabolic processes (p < 1 × 10⁻⁷). Of these genes, 88% are repressed by the TNF-α, hypoxia, or cotreatment model (Figure 2C); those that are repressed by TNF-α are most enriched in fat cell differentiation processes, whereas those repressed by hypoxia and cotreatment are most enriched in oxidation-reduction reactions (Table 1). Of these 352 genes, 13% are captured only by the cotreatment model (Figure 2C). These are most enriched in the cellular component mitochondria, suggesting that the cotreatment model recapitulates the mitochondrial dysfunction during insulin resistance.

The 298 adipogenesis-repressed DIO-induced genes are enriched in cell-cycle-related categories such as M phase (p = 1.9 × 10⁻²⁸) and DNA replication (p = 5.3 × 10⁻¹³) and inflammation-related processes such as cellular response to stress (p = 5.8 × 10⁻¹³) and chemotaxis (p = 0.03). Of these genes, 54% are recapitulated in the TNF-α, hypoxia, or cotreatment models (Figure 2D). The TNF-α model captures the upregulation of immune response and chemotaxis genes, whereas the hypoxia model captures the expression of genes related to cell-cycle processes. Importantly, the cotreatment model captures the main feature of both the TNF-α and the hypoxia models (Table 1).

**Systemic Transcriptome Changes in Adipose Insulin Resistance Revealed by Global Pathway Analysis**

To better visualize the data and to identify groups of genes that set the different models apart, we carried out principal component analysis (PCA) of the adipogenesis-related genes across the eight different models. PCA is a standard technique for reducing the dimensionality of data sets involving a large number of measurements while retaining as much variability as possible. The first principal component (PC1) explains 35% of the variance of the expression changes among the models. Projecting each data set along this axis reveals that the mouse models are well separated from the in vitro models (Figure 3A). Of the in vitro models, cotreatment is the closest to the mouse models. Genes making the most contribution to define PC1 are enriched in M
phase, chemokine activity, fat cell differentiation, and various lipid metabolic processes (Table 2). The second principal component (PC2) captures 18.5% of the data set variance, with the TNF-α, dexamethasone, and high-insulin models being closest to the mouse models; however, genes that contribute most to define PC2 are not enriched in any particular categories.

We repeated the PCA at a genome-wide level by using all 13,043 genes with FPKM >0.1. PC1 from the genome-wide PCA explains 21% of the data set variance. It separates the mouse models from the cell line models, and once again, the cotreatment model is closest to the mouse models (Figure 3A). Interestingly, although we include ten times more genes in the genome-wide PCA, the genes that contribute most to define the genome-wide PC1 are enriched in similar GO categories as those that define the adipogenesis-related PC1 (Table 2). This suggests that the set of 1,319 adipogenesis-related genes is able to capture many of the genome-wide differences of the different models.

To systematically analyze pathway changes that occurred during adipose insulin resistance, we searched for differences in expression of pathways defined in the Reactome and KEGG databases using gene set enrichment analysis (GSEA) of the genome-wide expression data (Subramanian et al., 2005). For a similar analysis based on the set of adipogenesis-related genes, see Table S4. Plotting the enrichment scores for each condition in a heatmap reveals pathways that are upregulated or downregulated in the different insulin-resistance models (Figure 3B). Pathways that are consistently downregulated in vivo include various glucose, lipid, and amino acid metabolic pathways as well as several cytochrome detoxification-related
pathways. The in vivo downregulation of metabolic pathways is largely captured by the TNF-α, hypoxia, and the cotreatment models, but not the other two models (Figure 3B). It is noticeable that whereas these models capture the direction of change (i.e., downregulation) of these pathways, the extent of downregulation in the in vitro models is not as significant as that in the DIO mouse models, an example of which is illustrated in the KEGG valine, leucine, and isoleucine degradation pathway (Figure S3).

The heatmap also reveals some consistently upregulated pathways in vivo, many of which relate to inflammatory responses, which are mainly captured by the TNF-α and cotreatment models and, to a lesser extent, by the hypoxia and high-insulin models (Figure 3B), whereas dexamethasone treatment downregulates many of these immune-related pathways. These analyses suggest that the systematic pathway changes occurring during insulin resistance vary in the different in vitro models, with the TNF-α and cotreatment models capturing many of these key changes.

One of the most salient features of the TNF-α and cotreatment models is that they appear to mimic the downregulation of key metabolic pathways and the upregulation of immune-related responses in vivo. Adipose tissue is a heterogeneous tissue comprising multiple cell types. Upon high-fat feeding, there is massive infiltration of activated macrophages into white adipose tissue (Weisberg et al., 2003). Because our comparison was made between multiple in vitro adipocyte models and in vivo whole adipose tissue, it is uncertain if our in vitro models were capturing the upregulation of the various inflammatory processes in the adipocytes or the associated macrophage-enriched stromal vascular fraction (SVF). In order to tease out the contribution of the different cell types in adipose tissue, we isolated adipocytes and SVFs from epididymal fat pads of

### Table 1. GO Analysis of Genes that Undergo Antiadipogenesis Transcriptome Shift in the Different In Vitro Models

<table>
<thead>
<tr>
<th>Gene Repression Is Captured by</th>
<th>Total (%)</th>
<th>Enriched GO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Of the 352 Adipogenesis-Induced DIO-Repressed Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α (a+ab+ac+abc)</td>
<td>61</td>
<td>mitochondrion (3.6 \times 10^{-15})</td>
</tr>
<tr>
<td>Hypoxia (b+ab+bc+abc)</td>
<td>38</td>
<td>mitochondrion (1.7 \times 10^{-15})</td>
</tr>
<tr>
<td>Cotreatment (c+ac+bc+abc)</td>
<td>74</td>
<td>mitochondrion (4.3 \times 10^{-30})</td>
</tr>
<tr>
<td>TNF-α+cotreatment (ac)</td>
<td>28</td>
<td>mitochondrion (4.1 \times 10^{-30})</td>
</tr>
<tr>
<td>Hypoxia+cotreatment (bc)</td>
<td>6</td>
<td>mitochondrion (4.3 \times 10^{-4})</td>
</tr>
<tr>
<td>TNF-α+hypoxia+cotreatment (abc)</td>
<td>25</td>
<td>mitochondrion (4.1 \times 10^{-30})</td>
</tr>
<tr>
<td>Only cotreatment (c)</td>
<td>15</td>
<td>mitochondrion (1.8 \times 10^{-15})</td>
</tr>
<tr>
<td>None of TNF-α, hypoxia, or cotreatment (d)</td>
<td>13</td>
<td>mitochondrion (9.9 \times 10^{-3})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Of the 298 adipogenesis-repressed DIO-induced genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (a+ab+ac+abc)</td>
</tr>
<tr>
<td>Hypoxia (b+ab+bc+abc)</td>
</tr>
<tr>
<td>Cotreatment (c+ac+bc+abc)</td>
</tr>
<tr>
<td>TNF-α+cotreatment, but not hypoxia (ac)</td>
</tr>
<tr>
<td>Hypoxia+cotreatment (bc)</td>
</tr>
<tr>
<td>TNF-α+hypoxia+cotreatment (abc)</td>
</tr>
<tr>
<td>Only cotreatment (c)</td>
</tr>
<tr>
<td>None of TNF-α, hypoxia, or cotreatment</td>
</tr>
</tbody>
</table>

The different categories are indicated in Figures 2C and 2D. *p values were calculated by Fisher’s exact tests assessing the significance of overrepresentation. Representative top GOs for each category are shown. Multiple hypothesis testings were corrected by Benjamini-Hochberg correction. Some combinations were not shown because there was no significant enrichment.
age-matched normal chow-fed and DIO mice. Although we found, as expected, that the downregulation of Pparg, Glut4, and Adipoq mainly occurs in adipocytes, the upregulation of chemokines (Ccl9), metalloproteinases (Mmp2), and inflammatory cytokines (Tnf) occurs in both adipocytes and SVFs (Figure 3C).

### Identifying Key Regulators of Insulin Resistance

The diverse transcriptional patterns of the in vitro models suggest that different transcriptional regulators are active under these conditions. In order to identify these transcription factors, we used an unbiased strategy based on DNase I hypersensitivity followed by high-throughput sequencing (DNase-seq) (Hesselberth et al., 2009; Siersbæk et al., 2011) and computational analysis of the sequences of hypersensitive regions (Eguchi et al., 2008; Ling et al., 2010). Our DNase-seq data are in good agreement with the literature (Birney et al., 2007; Mikkelsen et al., 2010; Siersbæk et al., 2011) at well-studied loci (Figure 4A).

Because our DNase-seq data for TNF-α-induced insulin resistance were of particularly high quality, we examined it in the greatest detail. MACS analysis (Zhang et al., 2008) identifies regions that lose or gain DNase hypersensitivity after TNF-α treatment (examples are shown in Figure 4B). Genes near regions with altered DNase hypersensitivity are more likely to
be differentially expressed ($p < 2.2 \times 10^{-16}$) (Figures 4C and S4). The observed correlation between changes in DNase hypersensitivity and gene expression suggests that hypersensitive sites may represent loci where there is a gain or loss of regulator binding. Motif analysis of these regions (see Experimental Procedures) identified a number of potential regulators in each condition, including, as expected, NF-κB and AP-1 for TNF-α treatment, glucocorticoid receptor (GR) for dexamethasone treatment, and hypoxia-inducible factor (Hif) in hypoxia (Tables S5 and S6).

Using both previously reported chromatin immunoprecipitation (ChIP) data and new experiments, we were able to confirm several hypotheses emerging from the motif analysis. To test our hypothesis that PPARγ regulates TNF-α-repressed genes, we examined previously reported PPARγ-binding data (Mikkelsen et al., 2010). Indeed, PPARγ-bound sites lose hypersensitivity in the TNF-α-treated cells ($p = 8.44 \times 10^{-10}$) (Figure 4D). By contrast, DNase hypersensitivity did not change at E2F4-bound regions ($p = 0.49$) (Figure 4D) (McLysag et al., 2010). Thus, it appears that PPARγ may be an important regulator of TNF-α-repressed genes.

To test the hypothesis that changes in hypersensitivity can be used to predict an increase in regulator binding, we carried out a p65 ChIP-seq experiment on the TNF-α-treated cells. We found that close to 60% of the high-confidence p65-bound sites ($p < 1 \times 10^{-10}$; 260 out of 437) overlap with the TNF-α-induced DNase-hypersensitivity regions. Examples of p65-bound genes include Ccl2, Ccl7, Saa3, Hpo, Lcn2, etc.; many of which are well-known targets of NF-κB. The average DNase-hypersensitivity profile around p65-bound sites increases in the TNF-α-treated cells ($p = 1.3 \times 10^{-1}$) compared to the control (Figure 4D). It is noteworthy that the C/EBP motif is highly enriched at p65-bound sites (Figure 4E; Table S7). This observation suggests that one or more members of the C/EBP transcription factor family are not only potential regulators of TNF-α-induced insulin resistance but use some of the same regulatory sites as p65.

**C/EBPβ in TNF-α-Induced Insulin Resistance**

Having shown that the C/EBP motif is enriched in regions with increased DNase hypersensitivity near the TNF-α-induced genes, we focused our analysis on Cebpb, which increased in expression to a relatively high level after TNF-α treatment (Figure 5A). We confirmed the increased expression using qPCR (data not shown) and western blots (Figure 5B). C/EBPβ protein expression is also higher in white adipose tissue harvested from mice fed a high-fat diet compared to mice fed a normal chow diet (Figure 5C). To assess if C/EBPβ binds to the regulatory regions of TNF-α-induced genes, we carried out C/EBPβ ChIP experiments on selected loci with increased DNase hypersensitivity near TNF-α-induced genes. We observed a significant increase in binding of C/EBPβ in the TNF-α-treated cells over control at the regulatory regions of Lcn2, Socs3, Glut1, and Il15, but not at the control region (Alb) (Figure 5D). To assess whether knocking down Cebpb would affect the gene induction of the above-mentioned genes after TNF-α treatment, we used two different siRNA constructs to knock down expression of this protein (Figure 5E). Upon Cebpb knockdown, there was a significant reduction in TNF-α-mediated induction of Lcn2, Ilr2, and Il15 (Figure 5E), indicating that Cebpb indeed is required for induction of genes following TNF-α treatment.

**DISCUSSION**

We have presented a detailed global transcriptome analysis of five different in vitro insulin-resistance models and compared them with three independent DIO mouse models. Our results show that different models capture distinct aspects of the in vivo changes. We find specific pathways that are altered in vivo and are captured by the individual models, and we are able to identify several transcriptional regulators that are likely to drive these changes.

It is not surprising that no single in vitro model captures all the features of DIO adipose insulin resistance, which are complicated phenotypes depending on multiple factors (e.g., mouse strain, high-fat diet formulation, and duration of high-fat feeding). Nevertheless, the TNF-α and hypoxia models, and even more so the cotreatment model, are able to recapitulate a wide range of the DIO transcriptional changes associated with metabolism. The impairment of metabolic pathways is not limited to the relatively well-studied glucose and lipid metabolic pathways. For example, the cytochrome P450 metabolic pathways are downregulated in vivo and in the TNF-α, hypoxia, and cotreatment models. White fat was suggested to have a prominent detoxification function (Fornet et al., 2009), and our analysis suggests that this function may be impaired in DIO mouse adipose tissue and also in these in vitro models. Moreover, we observed downregulation of branched-chain amino acid catabolic pathways in vivo and in the TNF-α, hypoxia, and cotreatment in vitro models, but not in the high-insulin or dexamethasone models. Levels of branched-chain amino acids (valine, leucine, and isoleucine) are elevated in obese (Newgard et al., 2009).
and diabetes-prone (Wang et al., 2011) humans. In addition, oxidation enzymes for branched-chain amino acids are downregulated in adipose tissue of obese and insulin-resistant humans (Pietiläinen et al., 2008). Our study highlights the downregulation of these pathways in DIO mice and shows that the TNF-α, hypoxia, and cotreatment models capture downregulation of these pathways.

Furthermore, our studies identify features captured by the five in vitro models uniquely or jointly. For example, the TNF-α and the cotreatment models capture the dedifferentiation-, chemotaxis-, and inflammation-related features that are observed in vivo. In particular, we found that the two TNF-α-related models are the only models among the five that can mimic the upregulation of genes related to chemotaxis. Indeed, three chemotaxis genes upregulated by TNF-α (Ccl2, Ccl7, and Ccl9) are among the six chemotactic factors that are consistently upregulated in adipose tissue, and predominantly adipocytes, of ob/ob and DIO mice (Jiao et al., 2009). The chemotactic nature of DIO adipocytes is suggested to contribute to macrophage infiltration and the ensuing chronic inflammatory responses (Weisberg et al., 2003). As for the inflammatory responses associated with insulin resistance, the TNF-α and cotreatment models are also largely able to replicate these. Conversely, hypoxia, high insulin, and dexamethasone are not
good models to capture the inflammation-related aspect of adipose insulin resistance. Given that dexamethasone is anti-inflammatory in nature, this is not surprising. However, it is rather unexpected that hypoxia could not model the DIO-induced inflammatory responses well because adipose hypoxia has been associated with an increase in expression of many inflammatory genes and the activation of NF-kB and TNF-a (Ye et al., 2007). We are confident that our hypoxic treatment was working because various hypoxia-responsive genes (e.g., Glut1, heme oxygenase 1 [Hmox1], pyruvate dehydrogenase kinase 1 [Pdk1], and vascular endothelial growth factor A [Vegfa]) were markedly upregulated in the hypoxia-treated cells; however, we cannot rule out that a longer duration of hypoxic treatment (>24 hr) is required to trigger the inflammatory responses in vitro.

Although the TNF-a and the cotreatment models capture the various immune response-related features, the high-insulin model and, to a lesser extent, the hypoxia model capture the up-regulation of genes related to cell-cycle processes and mitosis. This is in agreement with a recent study comparing gene expression of adipose tissue from insulin-resistant and insulin-sensitive subjects with matched BMI (Elbein et al., 2011), in which many
genes related to cell-cycle progression and cell adhesion were differentially expressed.

In our analysis, dexamethasone appears to be the model that is the least relevant to DIO adipose insulin resistance at the transcriptional level. However, we cannot exclude the possibility that dexamethasone induces proteomic changes that are similar to those in vivo. It is also plausible that the dexamethasone model is a better model for capturing features of insulin resistance of a different origin, such as insulin resistance associated with Cushing’s syndrome.

One important in vitro model of insulin resistance that we did not investigate in detail is fatty acid-induced insulin resistance (Van Epps-Fung et al., 1997). The conditions that we tested (800 μM of palmitate for 24–48 hr) induce only a minor impairment of insulin stimulation of glucose uptake and AKT phosphorylation, expression changes in ~100 genes (Figure S5), and enrichment in a limited number of gene sets (Table S4). Besides analyzing the transcriptional profiles of the diverse models of adipose insulin resistance, we explored in detail the transcriptional regulation of TNF-α-induced insulin resistance by combining genome-wide RNA-seq with DNase-seq analysis. In addition to known regulators such as PPARγ and NF-κB, we found that C/EBPβ is also a potential mediator of TNF-α-induced insulin resistance. Whole-body C/EBPβ deletion protects against obesity and insulin resistance upon high-fat diet treatment (Millward et al., 2007) and reduces adiposity and hepatic steatosis in db/db mice (Schoeder-Gloeckler et al., 2007). C/EBPβ has been extensively studied in the context of adipogenesis (Steger et al., 2010; Siersbaek et al., 2011); however, its role in TNF-α-induced insulin resistance has not been explored. We show that C/EBPβ protein expression increases upon TNF-α treatment and high-fat diet feeding and that it binds to the regulatory regions of several induced genes in TNF-α-treated 3T3-L1 cells. Importantly, induction of several TNF-α-responsive genes is diminished upon Cebpb knockdown. Furthermore, DNA motif analysis suggests that AP-1-related motifs are enriched in regions with increased hypersensitivity after TNF-α, dexamethasone, and hypoxia treatments. Activation of the transcription factor AP-1 is downstream of the activation of JNK, giving rise to the possibility that JNK activation is a common feature of multiple forms of insulin resistance.

We have shown that analysis of the mouse adipocyte DNase-seq data enables identification of known and novel regulators of gene expression. See the Extended Discussion for more information. In order to make this resource more broadly available, we have launched a web-based software: AdipoSight (http://fraenkel.mit.edu/adipo_sight/). Based on a list of user-supplied genes, the software will identify enriched DNA sequence motifs in the DNase-hypersensitive regions in the proximity of the genes (see Experimental Procedures).

In conclusion, our study highlights the particular features that the five in vitro models capture. This comprehensive and accurate description of the transcriptome changes of the five 3T3-L1 insulin-resistance models will be a rich resource for future studies.

EXPERIMENTAL PROCEDURES

In Vitro Cellular Insulin-Resistance Models
Cells were washed with PBS and changed to serum-free, low-glucose (1 g/l) DMEM with 0.5% BSA. Insulin resistance was induced with one of the following: 2.5 nM of TNF-α (R&D Systems) for 24 hr; incubation in a 1% oxygen chamber (Powers et al., 2010) for 24 hr; treatment with both 2.5 nM TNF-α and 1% oxygen for 24 hr; 1 μM dexamethasone (Sigma-Aldrich) for 24 hr; 100 nM insulin (Sigma-Aldrich) in high-glucose (4.5 g/l) medium for 24 hr; or 800 μM of palmitate (dissolved in 70% ethanol) for 48 hr in DMEM containing 1% serum and 2% BSA.

RNA-Seq Library Preparation, Sequencing, and Analysis
RNA-seq experiments were performed on biological triplicates. A total of 10 μg of total RNA was used for each RNA-seq library preparation according to the manufacturer’s instructions (Illumina). Quality of RNA was verified using Bioanalyzer (Agilent); only RNA with a RIN of >9 was used. Libraries were prepared and sequenced (Illumina; GAII) in a pair-end, 36 bp format, except for the cotreatment samples that were sequenced by Hi-seq in a single-end, 50 bp format. Reads from each sample were aligned to the mouse genome (mm9 build) using TopHat (version 1.1.0). Differential expression was quantified using Cuffdiff (Trapnell et al., 2010) (version 1.0.3). Differentially expressed genes are those that have a log2 fold change of >0.58 or <−0.58 and a q value of <0.05 when compared to the control condition. We also required that the differentially expressed genes used for downstream analysis have a FPKM greater than 0.1 in the control condition. Primers to verify RNA-seq results are listed in Table S8.

DNase-Seq
Intact nuclei were isolated from differentiated 3T3-L1 using a nuclei isolation kit (Sigma-Aldrich; NUC201) and prepared as described by Sabo et al. (2006). At least 30 million nuclei were used for each experiment; 50 μl/ml of DNase I (Promega RO1 RNase-free DNase; lot number: 25308616) was used for digesting 10 M cells at 37°C for 2 min followed by a SDS- and EDTA-based stop buffer. Digested nuclei were incubated at 55°C overnight with Proteinase K, extracted using phenol chloroform, and the “2-hit” DNA fragments were isolated using a sucrose gradient. Isolated DNA fragments were purified, subjected to the standard Illumina library preparation, and sequenced using Illumina GAII. Thirty-six-base pair-sequenced reads were mapped to the reference genome mm9 using Bowtie. Differential DNase-hypersensitive regions were identified using MACS (Zhang et al., 2009) using a p value threshold of 1 × 10−5; treatment-induced DNase-hypersensitivity regions were called with the treated cells as foreground and the untreated control as background. Conversely, treatment-repressed DNase-hypersensitivity regions were called with the untreated control as foreground and the treated cells as background. For the TNF-α-, dexamethasone-, and hypoxia-treated samples, control DNase-seq data set 1 (control 1) was used. For high-insulin treatment, control DNase-seq data set 2 (control 2) was used. See the Extended Experimental Procedures for more information.

ACCESSION NUMBERS
The raw data for the RNA-seq, ChiP-seq, and DNase-seq experiments were deposited in Gene Expression Omnibus with the accession number GSE35724.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Discussion, Extended Experimental Procedures, five figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.039.

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