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New insights into adipocyte-specific leptin gene expression

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The adipocyte-derived hormone leptin is a critical regulator of many physiological functions, ranging from satiety to immunity. Surprisingly, very little is known about the transcriptional pathways that regulate adipocyte-specific expression of leptin. In a recent published study, we pursued a strategy integrating BAC transgenic reporter mice, *in vitro* reporter assays, and chromatin state mapping to locate an adipocyte-specific cis-element upstream of the *LEP* gene in human fat cells. Quantitative proteomics (stable isotope labeling by amino acids in cell culture, SILAC) with affinity enrichment of protein-DNA complexes identified the transcription factor FOSL2 as a specific binder to the identified region. We confirmed that FOSL2 is an important regulator of *LEP* gene expression *in vitro* and *in vivo* using cell culture models and genetic mouse models. In this commentary, we discuss the transcriptional regulation of *LEP* gene expression, our strategy to identify an adipocyte-specific cis-regulatory element and the transcription factor(s) responsible for *LEP* gene expression. We also discuss our data on FOSL2 and leptin levels in physiology and pathophysiology. We speculate on unanswered questions and future directions.

function, bone density, and immune biology.^{4,5} Recombinant leptin has been developed as a drug for treating insulin resistance in patients with lipodystrophy.⁶ Although early studies using leptin as a weight loss agent in human obesity proved disappointing,⁷ recent trials using leptin in combination with the pancreatic polypeptide amylin show some promise.⁸ There has also been interest in using leptin to improve insulin action in a weight-loss independent fashion; however, recombinant methionyl human (r-Met hu) leptin did not enhance insulin sensitivity in obese subjects with type 2 diabetes.⁹

In the two decades since leptin was first identified, over 20,000 papers have been written describing leptin's actions in physiology and pathophysiology. In stark contrast to the wealth of data regarding the functions of leptin, however, our knowledge about the mechanisms involved in the transcriptional regulation of leptin gene expression, particularly the basis for its adipocyte-specific expression, is rather limited. Leptin is regulated primarily at the transcriptional level, and a wide variety of physiological conditions and pharmacological agents have been shown to affect its expression, including fasting and feeding, insulin, glucocorticoids, thiazolidinediones and even leptin itself.^{4,10,11} These studies are summarized in Table 1.

Keywords: leptin, Fosl2, transcription, obesity, diabetes, adipose, SILAC, ChIP-Seq

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Introduction

Leptin is an adipocyte-derived hormone that regulates food intake and energy expenditure, acting primarily through the central nervous system.^{1–3} In addition to its well-established role in metabolism, leptin also affects physiological processes as wide-ranging as reproduction, thyroid

Transcriptional Regulation of *LEP* Gene Expression

Leptin (encoded by the *LEP* gene in humans, *Lep* in mice) is generally expressed in an adipocyte-specific fashion. Small amounts of leptin have been reported in brain, pituitary, trophoblast, stomach, mammary epithelial cells, liver,

Table 1. Summary of factors regulating leptin gene expression in adipocytes

Physiological conditions	References	Direction of effect
Fasting	11, 26, 35	↓
Feeding	11, 26, 35	↑
Obesity	37	↑
Adipocyte volume	34	↑
Pharmacological agents	References	Direction of effect
Insulin	26, 38, 39	↑
Glucose	40	↑
Glucocorticoids	38, 39	↑
Hexosamines	41–43	↑
Cytokines (TNF, IL-1 and LIF) and lipopolysaccharide (LPS)	44, 45	↓
β ₃ -adrenergic agonist (CL316,243)	35	↓
Leptin	46	↓
Thiazolidinediones (TZD)	10	↑
Transcription factors	References	Direction of effect
C/EBPα	17–19	↑
ADD1/SREBP1	20	↑
AP-2β	22	↓
FOSL2	27	↑

chondrocytes and muscle,^{4,12–14} but the physiological importance of this low level expression, if any, has not been determined.

The proximal promoters of the *LEP* gene in mouse and human have been characterized^{15,16} and a classic TATA box has been identified, as well as binding sites for C/EBP, Sp-1, GR, and CREB, and an E box element that may bind SREBP1c.^{17–20} Although a transgenic reporter driven by a 762 bp leptin proximal promoter cassette was able to drive gene expression in vivo, it showed lower levels of expression in adipocytes than in a variety of non-adipose tissues and was not affected by fasting, suggesting that this region is not responsible for adipose tissue specificity of leptin expression or physiological regulation of leptin gene expression.²¹ Recently, the transcription factor activator protein-2β (AP-2 β) has been shown to inhibit *LEP* expression by direct binding to the promoter.²² Various SNPs have been reported in the porcine leptin promoter that correlate with *Lep* expression^{23,24} and demethylation of specific CpG islands occurs during adipogenesis, associated with the onset of *Lep* expression²⁵

All these studies have been limited by the fact that they have focused on the proximal promoter and/or on pre-specified adipocyte transcription factors. In fact, several major obstacles have impeded progress in identifying transcriptional pathways responsible for regulation of adipocyte-specific leptin gene expression, including (1) unreliable methods for unbiased detection of distal cis-regulatory elements, (2) insufficient tools to identify an unknown transcription factor binding to a known cis-regulatory element, and (3) a lack of good cell culture models for studying leptin gene expression in vitro. It is worth noting that the workhorse model of adipocyte research, the 3T3-L1 cell line, expresses extremely low levels of *Lep*²⁶ and is thus unsuitable for these studies. Recent advances in all of these areas encouraged us to pursue the mechanism of adipocyte-specific leptin gene expression once again.²⁷

Identification of a cis-Regulatory Element for Adipocyte-Specific *LEP* Gene Expression

We utilized a multi-step approach to finding cis-regulatory elements that direct *lep* gene expression. First, we sought to

map such regions grossly, using BAC-transgenic reporter mice that express EGFP from the *Lep* translational start site. Analyzing multiple transgenic founders carrying BACs with varying lengths of *Lep* flanking sequences allowed us to narrow down the region required for adipose-specific expression and demonstrated that the three *Lep* exons, both introns, and 5.2 kb of 5' flanking sequence were sufficient to drive adipocyte-specific EGFP expression. Next, we performed fine-mapping in vitro, by “tiling” across the upstream flanking region and through both introns with PCR primers, to generate reporter constructs that we could test in cultured human adipocytes. These cells are derived from human adipose stromal cells (hASCs), which can be differentiated into mature adipocytes in vitro and produce high levels of leptin. This effort identified a single 1 kb region approximately 4.5 kb upstream of the *LEP* transcriptional start site (TSS) that acts as enhancer in mature adipocytes. Further deletion and point mutations allowed us to discover a 30 bp region that was required for enhancer activity. Interestingly, we had previously performed chromatin state mapping in human adipocytes in culture, part of which involved mapping histone modifications associated with active and poised enhancers.²⁸ The region we identified by reporter assays overlay an active enhancer region^{29,30} in our chromatin state maps (the only such place in the vicinity), which provided significant reassurance that we had identified a region of bona fide importance.

Identification of the Transcription Factor(s) for Adipocyte-Specific *LEP* Gene Expression

Once a relevant cis-regulatory element is found, it can be very difficult to identify the cognate transcription factor(s) that bind and activate it. The most common approach is to use computational motif finding to find sequences that suggest a particular factor. We tried this and found several interesting motifs for the androgen receptor (AR), peroxisome proliferator-activated receptor α (PPARα) and nuclear factor erythroid 2-related factor 1 (NFE2L1). Unfortunately, functional

studies (i.e., overexpression and RNAi experiments) failed to demonstrate that any of these predicted factors were important in leptin expression. We therefore used our identified 30 bp cis-regulatory element as bait in an unbiased mass spectrometry-based quantitative proteomics approach combining stable isotope labeling by amino acids in cell culture (SILAC) with affinity enrichment of protein-DNA complexes using biotinylated DNA as affinity baits.³¹ To add specificity, we performed the assay in cultured human adipocytes as well as murine adipocytes differentiated from embryonic fibroblasts, both of which express leptin. Several transcription factors were found in each model, but FOSL2 and JUND were the only ones to appear in both. FOSL2 and JUND are both members of the AP1-transcription factor family, which includes FOS, FOSB, FOSL1, JUN, JUNB and JUND. We demonstrated that FOSL2 binds to our enhancer in mature human adipocytes, but not in pre-adipocytes, using ChIP-PCR.

FOSL2 is an Important Regulator of *LEP* Gene Expression In Vitro and In Vivo

To demonstrate the functional importance of FOSL2, we knocked it down with RNAi in human adipocytes; as predicted, this led to reduced *LEP* gene expression. Unfortunately, *Fosl2* global knockout mice die shortly after birth and before most adipose development is complete, so leptin levels in these animals cannot be interrogated. We did look at *Fosl2* heterozygous mice, which have normal adiposity however, and they show reduced circulating leptin, as do mice with adipocyte-specific deletion of *Fosl2*. In addition, osteoblastic precursor cells taken from the skulls of global *Fosl2* knockout mice can be differentiated into adipocytes in vitro; these cells display lower *Lep* gene expression. Gain-of-function studies were a bit harder to perform, in that overexpression of Fosl2 in mature adipocytes did not affect leptin expression. However, forced expression of Fosl2 during the development process resulted in adipocytes with normal overall differentiation but elevated leptin expression. Similarly, osteoblastic

precursors from *Fosl2*-transgenic mice showed higher *Lep* expression when differentiated into adipocytes in vitro. Since the *Fosl2*-transgenic mice suffered from systemic fibrosis and inflammation,³² metabolic studies of these animals could not be conducted. It appears that Fosl2 is both necessary and sufficient for leptin expression in adipocytes, but it must be present during the differentiation process to exert its effects. This implies that Fosl2 has both direct and indirect actions on the *Lep* gene.

Roles of FOSL2 in Regulating Leptin Levels in Physiology and Pathophysiology

As mentioned above, apart from being expressed in adipocytes, *Lep* mRNA synthesis in adipocytes is regulated by a variety of physiological and experimental stimuli. In mice, there are depot-specific differences in *Lep* mRNA content, with inguinal fat expressing higher leptin levels than retroperitoneal or epididymal adipose tissue.³³ There also appears to be a direct relationship between adipocyte volume and *Lep* expression regardless of depot.³⁴ In our study, a correlation of increased levels of *LEP* gene expression with increased levels of *FOSL2* gene expression was observed during the course of differentiation of human adipocytes and in different adipose depots in mice. In addition, we showed that stimulation of mature human adipocytes with dexamethasone resulted in increased *LEP* expression and simultaneous rise in *FOSL2* expression.

Fasting and Feeding Regulation of Leptin

One of the most important physiological functions of leptin is the regulation of fasting-feeding behavior.¹ Fasting reduces *Lep* mRNA and feeding increases it.³⁵ We measured *Fosl2* mRNA during the fed state and after 24 h of fasting, but we did not detect any significant differences. It is very possible that the mechanisms regulating adipocyte-specific gene expression of *Lep* are different from the mechanisms which regulate other physiological functions of *Lep*, such as fasting and feeding. It

is worth pointing out that our approach was geared toward the discovery of regulators of adipocyte-specific expression during differentiation, so it is perhaps unsurprising that Fosl2 might be not a regulator of the fasting-feeding response.

Roles of FOSL2 in Regulating Leptin Levels in Obesity

To determine if Fosl2 plays a role in the elevated *Lep* expression observed in obesity, we analyzed Fosl2 expression in different mouse models of obesity. In mice with diet-induced obesity as well as in mice with genetic obesity (*db/db*), we found that increased *Lep* gene expression was accompanied by increased *Fosl2* levels. A gene expression analysis of samples of subcutaneous fat from a human cohort with varying BMIs confirmed this association in humans.

Conclusions and Unanswered Questions

Several issues, however, remain unresolved. First of all, we do not yet understand how FOSL2, which is not tissue restricted to adipocytes, can regulate adipose-specific gene expression. There are several intriguing possible mechanisms, many of which are known to be employed by members of the AP-1 transcription factor family, to which FOSL2 belongs. These include differential expression, composition and orientation of the heterodimeric binding partners, post-translational modifications, and interaction with ancillary proteins.³⁶ Interestingly, although we identified JUND as a potential binding partner on our 30 bp cis-regulatory element, we could not demonstrate a specific requirement for this or any other Jun isoform in *Lep* expression. Perhaps there is greater functional redundancy among the Jun proteins than between FOS, FOSL1 and FOSB, which apparently cannot compensate for the loss of FOSL2 in leptin expression. Other factors may also be acting in concert with FOSL2. For example, there is a bona fide PPAR γ binding site at the 5' end of the 1 kb cis-regulatory region we discovered. Perhaps PPAR γ plays a role in enabling FOSL2 binding during adipogenesis.

Our results should by no means be taken to exclude a possible role for other cis-regulatory regions important for *Lep* expression. Our BAC transgenic results suggest that multiple regions, especially at the 3' end of the *Lep* gene, may be involved as well. For example, truncation of the 3' flanking sequence in our BAC transgenic reporter mouse model led to a significant decrease of EGFP expression in

adipose tissue and increased EGFP expression in other tissues. Taken together, these data suggest that many genomic regions and transcription factors will eventually be shown to regulate leptin expression.

Our study provided one of the first clues into the transcriptional regulation of the medically important adipokine leptin. Despite the fact that leptin levels are primarily regulated at the transcriptional

level, our understanding of the factors at play has been poorly developed. We utilized an integrated strategy that combined BAC transgenesis, human reporter assays, epigenomics, and unbiased quantitative proteomics to identify FOSL2. We believe that a similar multi-pronged approach will be required to tackle many of the remaining problems in tissue-specific transcriptional regulation.

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