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BRIEF REPORT

Adamts1 responds to systemic cues and gates adipogenesis

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ABSTRACT
Intuitively, excess caloric intake causes adipose tissue expansion. However, the signals and mechanisms by which this systemic trigger directs a local response in the adipose tissue are incompletely understood. Both hypertrophy of existing adipocytes and the generation of new adipocytes through differentiation of adipocyte precursor cells (APCs), contribute to adipose tissue expansion in response to changes in the diet. *Ex vivo* studies of this process elucidated an elegant network of mostly transcription factors that drive APCs through the differentiation (adipogenesis) process. Here we discuss our study that identified an *Adamts1* signal as a glucocorticoid and diet responsive regulator of an extracellular relay system that modulates the initiation of this intracellular adipogenesis program in APCs. Furthermore, we describe how we applied sensitive tools that enable monitoring of endogenous APC activity to study the early response to high-fat diet *in vivo*.

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Introduction

Changes in dietary intake can have a major impact on adipose tissue.[1,2] High-calorie diets that exceed the total energy expenditure of the body lead to increased adipose tissue mass. As excess caloric intake in the diet is the leading cause of obesity,[3] studying this process holds promise for revealing novel insights into the pathogenesis of obesity that enable the development of new approaches to address this major health problem.

Adipose tissue can expand by both hypertrophy of mature adipocytes and by the generation of new adipocytes through adipogenesis of precursor cells.[1] Both of these processes have been studied extensively but there are particularly significant barriers to examining adipogenesis *in vivo*. These obstacles include the difficulties of studying the systemic nature of the signals that trigger adipogenesis as well as technical hurdles of identifying, monitoring and tracing endogenous precursor cells in the adipose depots. As a result, adipogenesis has primarily been studied *in vitro* using pharmacological stimulation of either immortalized cell-lines that attempt to model true precursor cell biology or heterogeneous stromal vascular fractions harvested from adipose tissue depots.[4] Using these approaches, a large number of studies have led to the generation of a detailed molecular map of the intracellular cascade of events that propel adipocyte precursor cells through the differentiation process.[5] However, there remains a substantial knowledge gap in our understanding of how this differentiation cascade is physiologically gated and triggered *in vivo*.

Glucocorticoids, particularly dexamethasone, are commonly used as a critical component of a cocktail of pharmacological agents that induce adipogenesis *in vitro*.[6] Glucocorticoids are also frequently used *in vivo* because of their efficacy in treating a broad array of medical conditions. However, one of the most common side effects of systemic glucocorticoids, when used for extended periods of time or at high-doses, is the induction of obesity[7] suggesting that excess glucocorticoids promotes adipogenesis even if they are physiologically dispensable for this process.[8] Because of these *in vitro* and *in vivo* properties, we elected to use glucocorticoids as medically relevant molecular probes to reveal molecular mechanisms that regulate adipose tissue expansion.

Our studies also apply recent advances in the stem cell biology of adipocyte precursor cells (APCs). Even though using primary endogenous APCs, rather than cell lines or heterogeneous populations of surrogate cells, is time-consuming and technically demanding, we believe that the rationale that using this approach will more clearly

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define physiologically relevant pathways and modes of regulating adipogenesis is compelling. While it remains unresolved if there are multiple distinct APC populations, we feel there are robust data from multiple independent laboratories that the population of Lin(-):CD29(+):CD34(+):Sca-1(+) cells, isolated from adipose depot are *bona fide* APCs. Data on this population of APCs, which include their ability to reconstitute an adipose depot *in vivo*[9] and lineage tracing studies,[10,11] provide a foundation for studying adipogenesis *in vivo*.

Results

Using glucocorticoids and monitoring endogenous APCs, we interrogated how systemic cues communicate a signal to initiate adipogenesis *in vivo*.[12] We discovered that increasing circulating glucocorticoid levels by exogenous administration of dexamethasone inhibits the expression of the *Adamts1* gene in mature adipocytes. Furthermore, we found that *Adamts1* expression leads to the production of ADAMTS1 protein, which is then secreted from the mature adipocytes. Our data indicate that extracellular ADAMTS1 signals to other adipocytes, resulting in the induction of secretion of PTN protein. PTN, again acting as an extracellular protein, signals to APCs and inhibits adipogenesis by modulating Wnt signaling. When we generated transgenic mice that constitutively overexpress *Adamts1* in adipocytes (*Adam Tg*), we found that these animals have smaller adipose depots compared to wild-type littermates. Furthermore, we confirmed that overexpression of ADAMTS1 in *Adam Tg* mice is sufficient to block the effects of glucocorticoids on APCs.

We interrogated the endogenous APCs in *Adam Tg* mice using EdU pulse-chase experiments to monitor the levels of adipogenesis *in vivo*.[12] As mature adipocytes do not divide, EdU labeling of mature adipocytes can only occur from the differentiation of a pulse-labeled precursor cell and, therefore, the number of EdU positive mature adipocytes detected after a chase reflects the level of adipogenesis that occurred during that same interval. In this way, we established that *Adam Tg* mice have significantly lower rates of adipogenesis *in vivo* compared to wild-type littermate controls. In addition, we demonstrated that *Adam Tg* mice can be rescued from the block in adipogenesis phenotype, again using *in vivo* approaches: We found that injecting *Adam Tg* mice with an antibody that neutralizes PTN activity is sufficient to restore adipogenesis to wild-type levels. In addition to defining a critical pathway that regulates the initiation of the cell-intrinsic adipogenesis cascade in endogenous APCs *in vivo*, these studies also establish that extracellular signaling is the essential mechanism by which this process is regulated.

Together, these data validate our approach of using medically relevant glucocorticoids to elucidate an *in vivo* mechanism for gating the initiation of adipogenesis. We were next inspired to test how this pathway might relate to physiological induction of adipogenesis in response to a high-calorie diet. The use of high-fat diet (HFD) is the most validated approach to study diet-induced obesity. Therefore, we tested if the ingestion of HFD impacts *Adamts1* signaling.[12] Importantly, we conducted these studies in wild-type mice to test for the physiological relevance of *Adamts1*. Indeed, we found that in adipose depots where HFD induces adipogenesis, *Adamts1* expression was also repressed by HFD. Next, to test if HFD-induced adipogenesis is dependent on the repression of *Adamts1*, we repeated the studies using *Adamts1 Tg* mice, where expression of *Adamts1* persists in adipose tissue irrespective of the ingestion HFD. Importantly, these studies revealed that the repression of *Adamts1* expression is essential for HFD-induced adipogenesis.

We were intrigued by the fact that a pathway regulated by exogenously administered glucocorticoids is essential for HFD-induced adipogenesis and speculated that endogenous glucocorticoids might have a role in the physiological regulation of *Adamts1* pathway in response to HFD. Given the depot-specificity of the induction of adipogenesis by HFD, we hypothesized that changes in glucocorticoid levels in response to HFD would be tissue specific, rather than systemic. Tissue-specific regulation of glucocorticoids is a well-established process mediated by context-specific expression of two enzymes (11BHSD1 and 11BHSD2), which activate and inactivate intracellular glucocorticoids respectively.[13] Remarkably, we found that ingestion of HFD leads to adipose depot-specific activation of the 11BHSD enzymes: Expression of the glucocorticoid activating enzyme 11BHSD1 is induced in depots where adipogenesis is induced by HFD and expression of the glucocorticoid inactivating enzyme 11BHSD2 is induced in depots where adipogenesis is not induced.[12] These data indicate that a pathway that is critical for diet-induced adipogenesis is embedded within physiological glucocorticoid signaling. Furthermore, the results illuminate a novel mechanistic paradigm by which depot-specific induction of adipogenesis is specified.

In addition to monitoring adipogenesis, our approaches enable us to interrogate the activity of the endogenous APC population. Specifically, by combining *in vivo* EdU pulse-chase with flow cytometery of the

APC population we elucidated the impact of *Adamts1* signaling on both adipogenesis as well as the rates of APC proliferation.[12] Because of the sensitivity of these techniques, we decided to extend these investigations of the adipose tissue and APC population to an early time-point after the initiation of HFD. Intriguingly, after just one month of HFD, we detected depot-specific changes in the *Adamts1* signal in adipose tissue in response to HFD (Fig. 1A, B). These changes were associated with depot-specific responses in the APC population: We detected an induction of adipogenesis in the perigonadal visceral adipose depot (gWAT) while in the subcutaneous inguinal depot (iWAT) the rate of proliferation of APCs was induced (Fig. 1C, D).

Discussion

In conclusion, we identified a diet responsive extracellular signal that directs APCs in the decision to differentiate. Our studies support the model that this depot-specific response to the HFD is coordinated by differential modulation of *Adamts1* and *Ptn* levels, leading to distinct activation of Wnt signaling in APCs. The extracellular nature of the ADAMTS1-PTN-Wnt pathway is particularly intriguing to us, as unraveling the implications for this mechanism will likely expose an important new understanding of systemic regulation of adipose tissue. We speculate that this extracellular pathway evolved as a means to keep the adipose depot size itself in homeostatic balance under basal conditions by

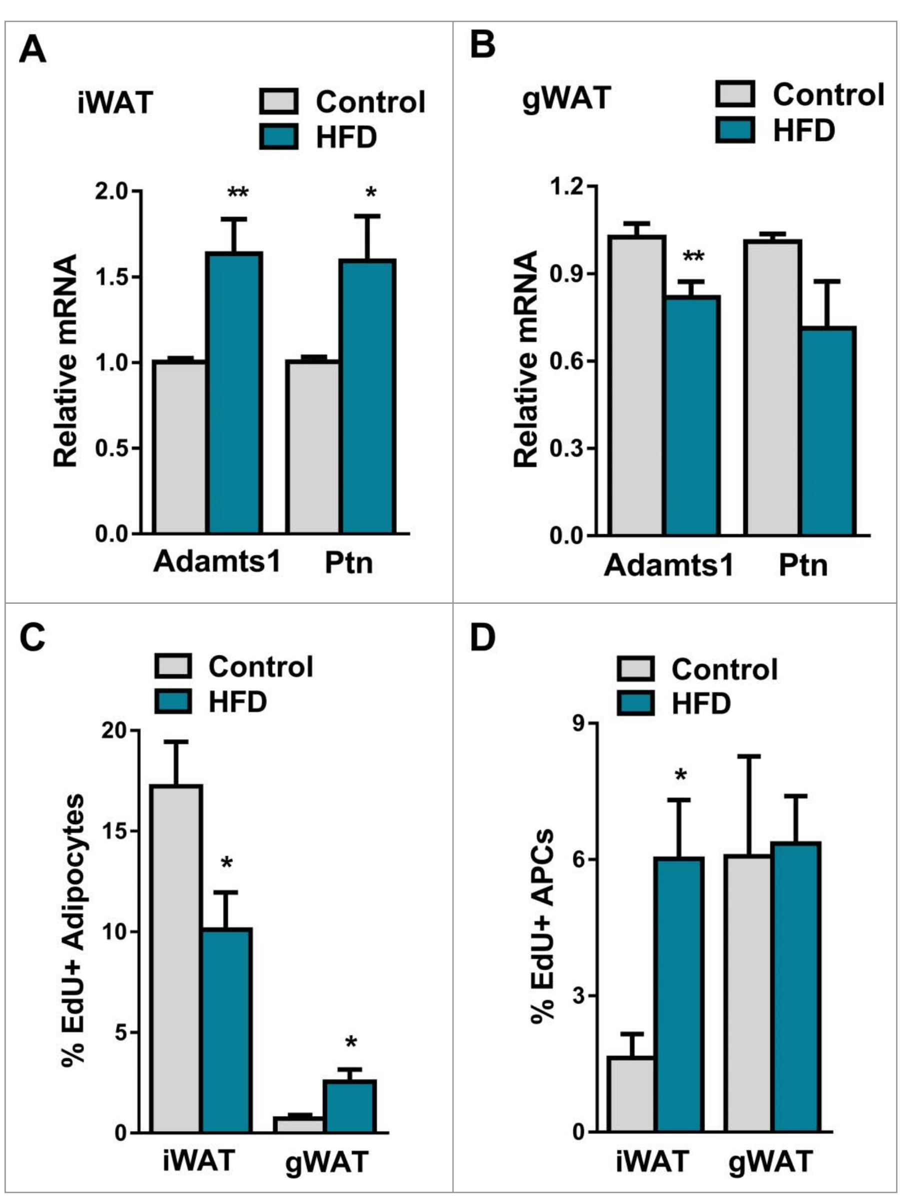

Figure 1. Depot-specific changes in response to 1-month of high-fat diet. qRT-PCR measuring the expression levels of Adamts1 and Ptn in the iWAT (A) and gWAT (B) adipose depots after one month of HFD compared to mice fed standard chow diet (Control). Flow cytometry of iWAT and gWAT adipose depots measuring the number of EdU positive mature adipocytes (C) and APCs (D) after a pulse-chase after one month of HFD.

the sum of competing and synergizing endocrine, paracrine and other systemic signals that serve to gate or balance any one trigger with inputs from additional cues. In addition, this system is able to maintain responsiveness to stimuli that summon adipose depot expansion when needed. Testing this hypothesis would include identifying counter-regulatory signals to the potent *Adamts1* signal that modulate APC activity. Furthermore, it will be of particular interest to elucidate if these putative counter-regulatory signals also converge on Wnt signaling or if entirely independent intracellular mechanisms are responsible for gating the initiation of differentiation in APCs.

An additional interesting feature of the *Adamts1* pathway we identified is the ADAMTS1 relay to induce PTN as a critical step for the pathway function. Strikingly, we found that inhibiting PTN with a neutralizing antibody completely rescued APCs from the APC proliferation and differentiation phenotype *in vivo* in the *Adamts1 Tg* mice. While the logic of embedding a relay system into the regulation of APCs is not fully apparent from our studies, we speculate that this enhances the coordination and feedback with the systemic environment. In support of this hypothesis, our data demonstrate that HFD results in a decrease in circulating levels of PTN in the mouse models and circulating PTN also decreased in humans participating in an overfeeding study.[12] Drawing a comparison with other regions of the endocrine system, the hypothalamus-pituitary-adrenal (HPA) axis appears to utilize a similar approach, with hormones secreted from the hypothalamus for a short-range stimulation of the pituitary gland to secrete secondary hormones that circulate and implement the effects.[14] As with the HPA axis, we hypothesize that the ADAMTS1-PTN relay controlling APCs, enables multilevel feedback loops that modulate the final signal.

In summary, we identified *Adamts1* as the modulator of a potent pathway that converts changes in diet into a cellular signal in adipose tissue that controls APC activity *in vivo*.[12] These findings reveal a previously unrecognized control mechanism for adipose tissue to respond to increased caloric intake by adjusting the rate of APC differentiation. Elucidating the pathways by which high-calorie diets generate a cellular signal governing adipogenesis could lead to the development of new approaches for the treatment and prevention of obesity and associated disorders such as diabetes.

Methods

Mice

All studies were approved by Stanford University's Administrative Panel on Laboratory Animal Care Committee. For the HFD experiments in wild-type mice, 10 week-old male C57BL/6J mice were purchased from Jackson Laboratories and fed a diet with 60% kcals from fat (Bioserv, F3282) ad libitum for 1 month.

In vivo *EdU labeling*

5-ethynyl-2'-deoxyuridine (EdU) (Carbosyth) was administered by intraperitoneal injections (100 mg/kg) once every day for 2 weeks. For flow cytometric analysis, SVF was prepared and stained for surface markers as previously described 12 using the following the antibodies: SCA1 APC (BioLegend, 108112), CD45 PE-Cy7 (eBioscience, 25-0421-82), CD31 PE-Cy7 (eBioscience, 25-0311-81). Samples were then fixed in 4% paraformaldehyde (PFA)/PBS for 30 minutes, and then permeabilized in 1x saponin-based permeabilization buffer (Invitrogen) for 10 minutes. EdU was labeled using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) by following the manufacturer's protocol. For adipocyte EdU-labeled nuclei analysis, adipocyte nuclei were isolated in cold nuclei extraction buffer (320 mM sucrose, 5mM $MgCl_2$, 10mM HEPES, 2% Triton X-100, pH 7.4) in a dounce homogenizer. Nuclei were centrifuged at 3000 X g for 15 min and washed with cold nuclei wash buffer (320 mM sucrose, 5mM $MgCl_2$, 10mM HEPES, 1% BSA and 0.1% sodium azide, pH 7.4). EdU nuclei were labeled using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit. DAPI (500 ng/mL) was added to cells or nuclei immediately prior to FACS analysis.

Disclosure of potential conflicts of Interest

Stanford University has filed a patent related to the discoveries described in this manuscript.

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