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Transcriptional fingerprinting of "browning" white fat identifies NRG4 as a novel adipokine

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Brown adipocytes help to maintain body temperature by the expression of a unique set of genes that facilitate cellular metabolic events including uncoupling protein 1-dependent thermogenesis. The dissipation of energy in brown adipose tissue (BAT) is in stark contrast to white adipose tissue (WAT) which is the body's primary site of energy storage. However, adipose tissue is highly dynamic and upon cold exposure profound changes occur in WAT resulting in a BAT-like phenotype due to the presence of brown-in-white (BRITE) adipocytes. In our recent report, transcription profiling was used to identify the gene expression changes that underlie the browning process as well as the intrinsic differences between BAT and WAT. Neuregulin 4 was categorized as a cold-induced BAT gene encoding an adipokine that signals between adipocytes and nerve cells and likely to have a role in increasing adipose tissue innervation in response to cold.

Keywords: adipokine, adipocyte, Brown fat, BRITE adipocyte, beige adipocyte, metabolism, neuregulin, Nrg4, thermogenesis, white fat

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Adipose tissue is a remarkably dynamic organ that responds to the external and internal environment. It is composed of discrete subcutaneous and visceral depots that contain different amounts of white adipocytes, specialized cells for energy storage, and brown adipocytes that serve to generate heat by thermogenesis. Some white adipose tissue (WAT) depots undergo profound changes, following acclimatization to cold temperatures that result in a phenotype characteristic of brown adipose tissue (BAT). The adipocytes within the white fat that display brown fat features, such as multilocular lipid droplets and inducible uncoupling protein 1 (UCP1) expression, are termed "brown-in-white" (BRITE) or beige adipocytes.[1] There has been a recent resurgence in the study of brown fat following the identification of functional deposits in adult humans.[2] With obesity becoming an increasingly important global health issue due to its associated risk for type 2 diabetes, cardiovascular disease, hypertension, and various cancers, it is imperative that medical researchers identify novel approaches to improve metabolic health. As BAT contributes to energy expenditure, recruitment and activation brown fat has great potential as a therapeutic target to combat weight gain.

Differential Gene Expression in BAT and WAT

The fundamental histological and functional differences between adipose tissues including the multilocular and unilocular adipocyte morphology of BAT and WAT, respectively, and the site of thermogenesis in BAT have been known for many decades.[3] The differential gene expression that underpins the key morphological and functional differences is yet to be fully elucidated. In a recent report, we utilized microarray technology to investigate intrinsic depot-specific differences in gene expression as well as the changes in response to environmental temperature.[4] Thus, we profiled gene expression of interscapular BAT, inguinal subcutaneous WAT and the visceral mesenteric WAT depots from animals acclimatized for 10 d to either warm (28°C) or cold temperatures (6°C). The warm temperature is near thermoneutrality for the mouse so BAT activity is minimal whereas BAT is fully activated in the cold to maintain body temperature. It is

clear that the discrete white fat depots respond very differently to the cold treatment with subcutaneous being highly responsive and the mesenteric being largely unresponsive. From our analysis were identified key depot-specific gene expression as well as cold-induced and genes that define the BRITE fat.

Adipose tissue gene profiling studies have been undertaken by other researchers although there are several inter-study design differences such as mouse strain, gender, temperature, and duration of acclimatization. To distil the congruent gene expression differences between BAT and WAT or induced by cold in WAT the gene lists from our work and other studies[5,6] were compared and the top common hits are listed in **Table 1**. This post hoc analysis reveals the transcriptional adaptations, in response to cold, and intrinsic differences between mouse depots that are similarly modulated despite differing experimental paradigms.

For the genes induced following cold exposure the classical BAT genes were present including Ucp1, Cidea, Cpt1b, and PPARα. The majority of the transcripts on this list are important for fatty acid metabolism with the most highly regulated being Elovl3, Slc27a2, and Fabp3. It is noteworthy that the genes identified are involved in both anabolic and catabolic processes. In addition, there are high levels of Pdk4, which phosphorylates and inhibits pyruvate dehydrogenase complexes, leading to decreases in the formation of acetyl-CoA and a greater flux of pyruvate toward oxaloacetate and the glyceroneogenic pathway.[7] This could be important at times when rates of lipolysis are high to maintain levels of glycerol-3-phosphate for fatty acid resterification to prevent depletion of the intracellular triglyceride pool.

Transcriptomic studies often identify expressed genes that have yet to been fully annotated or ascribed functions. One such cold-induced transcript is AI317395 which may have an important role in brown and BRITE adipocytes as analysis indicates it encodes a protein that is predicted to be a glucose transporter containing a major facilitator superfamily domain, general substrate transporter and

Table 1. Cross-study consistent gene expression changes in response to cold in WAT and enriched in BAT compared to WAT. Cold-regulated genes: Genes that were induced after cold acclimatization in WAT, in both GSE510804 and GSE134325 microarray studies. The data were accessed from NCBI's Gene Expression Omnibus26 (http://www.ncbi.nlm.nih.gov/geo/). BAT genes: Genes listed are more highly expressed in BAT vs Subcut WAT, in both GSE51080 and GSE440596 The top 25 genes common to 2 datasets are presented following comparison of significantly differentially expressed genes ranked by fold change

	Cold-regulated genes*	Function	BAT genes†	Function
1	Elovl3	fatty acid biosynthetic process[27]	Zic1	regulation of transcription, DNA-templated[28]
2	Ucp1	oxidative phosphorylation uncoupler activity[29]	Ucp1	oxidative phosphorylation uncoupler activity[29]
3	Slc27a2	long-chain fatty acid-CoA ligase activity[30]	Cpn2	enzyme regulator activity[31]
4	Fabp3	long-chain fatty acid transporter activity[32]	Fabp3	long-chain fatty acid transporter activity[32]
5	S100b	calcium-dependent protein binding[33]	Slc27a2	long-chain fatty acid-CoA ligase activity[30]
6	Acot11	fatty acid metabolic process[34]	9130214F15Rik	—
7	Cpt1b	carnitine O-palmitoyltransferase activity[35]	Kng1	negative regulation of peptidase activity[36]
8	Cox7a1	oxidation-reduction process[37]	Ppara	sequence-specific DNA binding transcription factor activity[38]
9	Gyk	glycerol-3-phosphate biosynthetic process[39]	Pank1	coenzyme A biosynthetic process[40]
10	Kng1	negative regulation of peptidase activity[36]	Cpt1b	carnitine O-palmitoyltransferase activity[35]
11	Otop1	Anti-inflammatory activity[41]	Me3	oxidation-reduction process[42]
12	PPARa	sequence-specific DNA binding transcription factor activity[38]	Plet1os (2310014F07Rik)	Non-coding RNA highly expressed in BAT, heart and skeletal muscle
13	Cpn2	enzyme regulator activity[31]	Cox7a1	oxidation-reduction process[37]
14	Pdk4	regulation of fatty acid biosynthetic process[7]	Gnas	G-protein β/gamma-subunit complex binding[9]
15	Cyp2b10	oxidation-reduction process	Gmpr	oxidation-reduction process[43]
16	Dio2	thyroid hormone metabolic process[44]	Myo5b	regulation of protein localization[45]
17	Pank1	coenzyme A biosynthetic process[40]	Acot11	fatty acid metabolic process[34]
18	Naglt1a (AI317395)	sodium-dependent glucose transporter (predicted)	Mapt	regulation of microtubule-based movement
19	Fbp2	gluconeogenesis[46]	Dio2	thyroid hormone metabolic process[44]
20	Aspg	lipid catabolic process[47]	S100b	calcium-dependent protein binding[33]
21	Esrrg	sequence-specific DNA binding transcription factor activity[48]	Ntrk3	transmembrane receptor protein tyrosine kinase signaling pathway[49]
22	Idh3a	oxidation-reduction process[50]	Esrrg	sequence-specific DNA binding transcription factor activity[48]
23	Slc25a20	Mitochondrial substrate/solute carrier[51]	Fbp2	gluconeogenesis[46]
24	4931406C07Rik	ester hydrolase activity	Tspan18	—
25	Cidea	Lipid metabolic process[13]	Otop1	Anti-inflammatory activity[41]

*The subcutaneous WAT gene lists were generated from data sets GSE51080 which used 10 week old female 129Sv mice acclimatized to either 28°C or 6°C for 10 d and GSE13432 which used 6–8 week old male C57BL/6 mice acclimatized to either 30°C or 4°C for 5 weeks. †For GSE51080, interscapular BAT and subcutaneous WAT was taken from 10 week old female 129Sv mice acclimatized to 28°C for 10 d For GSE44059 adipocytes were purified from interscapular BAT or subcutaneous WAT of young adult male C57BL/6 housed at 23°C.

is therefore termed sodium-dependent glucose transporter 1A (Naglt1a). High levels of glucose are taken up by BAT as illustrated by the application of positron emission tomography using fluorine-18 fluoro-2-deoxy-D-glucose to visualize this depot in humans.[2] The elevated expression of the Naglt1a transcript raises the possibility that it could contribute to glucose uptake by BAT.

Examination of the genes more highly expressed in interscapular BAT compared to subcutaneous WAT in both our study[4] and that of the Wolfrum group[6] reveals a considerable overlap with the cold-regulated genes (14 out of 25 genes) including the enrichment of transcripts associated with fatty acid metabolism (**Table 1**). The transcriptional regulator zinc finger protein of the cerebellum (Zic1) is the top differentially expressed gene suggesting a potential role in the differential gene expression profile. It may be pertinent that Gnas is among the transcripts most enriched in BAT. This is in agreement with a recent RNA-seq study that identified this transcript as having the highest number of reads of any mRNA in BAT of the 13-lined ground squirrel.[8] The gene encodes the stimulatory G-protein α subunit G_s-α. As β adrenergic receptors are the central mediators of BAT activation and coupled to G_s-α, the high expression of this G protein subunit may be integral to signal transduction in response to cold. Gnas is an imprinted gene[9] and it is noteworthy that several other imprinted genes are linked with adipose biology. For example, Dlk1 is key regulator of the transition from preadipocyte to mature adipocyte[10] and we found neuronatin to be one of the few genes more highly expressed in WAT compared to BAT.[4] Other relevant genes that are predicted to be imprinted include Prdm16, Zic1, and Bmp8b.[11]

In our study, we undertook a strategy to define the "BRITE" transcriptome. For this, we determined the genes that were enriched in both BAT versus subcutaneous WAT, and BAT vs. mesenteric WAT (comparisons at 28°C) as well as increased in subcutaneous WAT by cold exposure. This analysis confirmed the association of Ucp1, Cidea, PGC-1α, Plin5, PPARα, and Otop1 with the "browning" of adipose tissue and also reveals additional genes that are part of the brown/"BRITE" transcription fingerprint including the fatty acid receptor Gpr120, the regulator of G protein signaling Rgs7, the mitochondrial membrane Ca^{2+}/H^{+} antiporter Letm1, and signaling factor Nrg4.

Neuregulin 4 (Nrg4) an Adipokine Secreted by Brown Adipocytes

There is increasing evidence for an endocrine role of BAT. The secretion of factors including FGF21, VEGF-A, RBP4, FGF2, IL-1α, IL-6, IGF-1, BMP8B, and prostaglandins (reviewed in[12]) by BAT highlights its signaling capacity through endocrine, paracrine, and autocrine actions. We reported that NRG4 is a novel adipose tissue signaling factor that could have a key role in adipocyte-neuronal cross-talk.[4] This epidermal growth factor (EGF)-like factor was identified as a member of the group of genes that define the "BRITE" transcription signature. Importantly, although visceral mesenteric WAT gene expression was largely unresponsive to cold, Nrg4 was one of only 5 genes identified by microarray as increased at 6°C vs 28°C (the others being Ucp1, Orm3, Cabc1, and Acsf2). BAT is the tissue that expresses the highest level of Nrg4 mRNA although significant amounts are detectable in gonadal and subcutaneous WAT as well as mammary gland. This pattern is remarkably similar to the more extensively studied BAT genes such as Cidea, which encodes a lipid droplet-associated protein.[13] Furthermore, the expression of Cidea and Nrg4 is largely restricted to mature adipocytes with very low levels present in preadipocytes. The production of NRG4 by adipocytes is in contrast to other neurotrophic factors that primarily affect adipose tissue biology primarily through hypothalamic outflow pathways. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF) are examples of factors produced by the central nervous system that affect energy balance (Reviewed in [14]). NGF is however also produced by cultured brown adipocytes[15] with a potential role in adipocyte-neuronal communication. A neurotrophic role for NRG4 is supported by studies of the related NRG1 that found it affected the development of neuronal progenitor stem cells.[16]

Like the other members of the neuregulin family (NRG 1–4), NRG4 contains a transmembrane domain with an adjacent extracellular EGF-like domain at the NH_2-terminal.[17] This region contains proteolytic cleavage-sensitive sites that allow the release of the biologically active EGF-like domain. To date, Nrg4 has been studied predominantly in cancers and is reported to be overexpressed in advanced-stage prostate cancer[18] as well as a facilitating a survival signal in colon epithelial cells.[19] It has been demonstrated previously that NRG4, in conditioned medium from transfected Cos7 cells or a chemically synthesized and refolded peptide, can elicit neuronal outgrowth in PC12-HER4 cells (a cell line derived from a rat adrenal medullary pheochromocytoma and stably expressing the receptor for NRG4, ERBB4).[20] We found NRG4 protein was secreted by mature brown adipocytes, but not by brown preadipocytes.[4] One of the key differences between brown and white adipose tissue is the degree of sympathetic innervation.[21] Thus, NRG4 can be proposed as an important signaling factor that facilitates the innervation of adipose tissue. This is supported by the ability of brown adipocyte conditioned medium to promote neurite outgrowth by PC12-HER4 cells.[4] The effect was specific for NRG4, as neurite outgrowth was prevented in conditioned media from adipocytes in which it was knocked down by shRNA interference.

Other growth factors such vascular endothelial growth factor (VEGF) are reported to have important actions in brown adipose tissue. VEGF-A causes an increase in BAT thermogenesis and promotes the browning of WAT.[5] It has multiple actions to affect adipocyte biology including vasculogenesis, angiogenesis, control of vascular permeability, and the recruitment of M2 anti-inflammatory macrophages.[22] Of course the action of NRG4 is not necessarily restricted to adipocyte-nerve cell signaling and it may have other functions including autocrine actions particularly as Erbb4 is expressed in BAT and cultured brown adipocytes

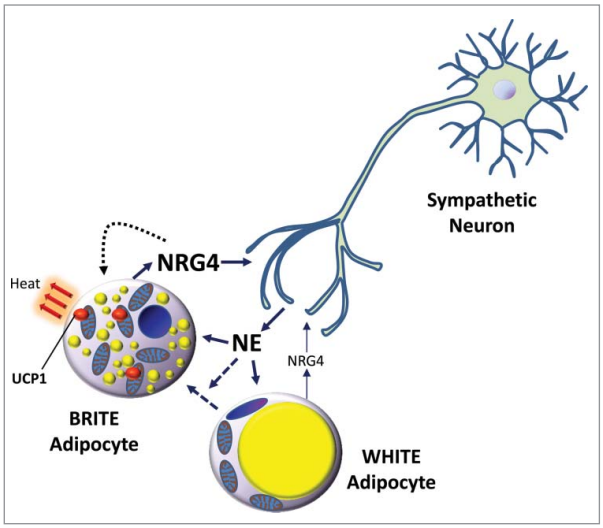

Figure 1. NRG4 is brown adipocyte adipokine that promotes neurite outgrowth. Neuregulin 4 (Nrg4) is more highly expressed in brown adipocytes compared to white adipocytes. Upon cold exposure, norepinephrine (NE) is secreted and activates brown fat as well as initiating the "browning" of white fat resulting in upregulated Nrg4 mRNA. NRG4 is secreted by brown adipocytes and can signal to neurons to promote neurite outgrowth. Thus, NRG4 is a brown/brown-in-white (BRITE) adipokine that has a potential role in enhancing sympathetic innervation of adipose tissues needed to activate thermogenic functions.

(MC unpublished observations). Dissection of the metabolic role of NRG4 has not been undertaken, but clues to its potential functions may be provided by studies in muscle where, for example, neuregulins are important for metabolism including glucose uptake.[17] In addition, NRG4 signaling may modulate a central coregulator of brown fat gene expression, in manner similar to NRG1 that leads to phosphorylation of PGC-1α and its activation in muscle cells.[23] As NRG4 is a growth factor, it may have paracrine effects to increase or maintain cell number during the cell turnover that may occur during adipose tissue remodeling from cold exposure. Alternatively, it could act to affect differentiation of preadipocytes as NRG1 acts to promote myoblast differentiation.[24,25] Additional studies including in vivo investigations are required to fully delineate the roles of NRG4 in BAT and determine its ability to control sympathetic nerve innervation of adipose tissues. It is noteworthy that a recent investigation identified adipose tissue-derived NRG4 as a key regulator of hepatic lipolysis.[52]

Following our analysis of mRNA expression in discrete adipose tissue depots we identified NRG4 as an adipokine primarily expressed by brown adipocytes that promotes neurite growth (**Fig. 1**) and therefore has the capacity to affect BAT sympathetic tone and facilitate thermogenic functions. As adult humans possess functional BAT NRG4 could be a new therapeutic target with the potential to increase BAT and BRITE adipocyte sensitivity to adrenergic signaling. The regulation of Nrg4 expression is only one example of the transcriptional events that underpin the WAT to BAT transition. Future investigations of the genes associated with the BRITE transcription signature could help develop new strategies to modulate energy balance for the treatment of metabolic disorders such as obesity and type 2 diabetes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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