

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

Mark Christian*

Division of Metabolic and Vascular Health; Warwick Medical School; University of Warwick; Coventry, UK

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*Correspondence to: Mark Christian; Email: M.Christian@warwick.ac.uk

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Commentary to: Rosell M, Kaforou M, Frontini A, Okolo A, Chan YW, Nikolopoulou E, Millership S, Fenech ME, MacIntyre D, Turner JO, Moore JD, Blackburn E, Gullick WJ, Cinti S, Montana G, Parker MG, Christian M. Brown and white adipose tissues. Intrinsic differences in gene expression and response to cold exposure in mice. *Am J Physiol Endocrinol Metab* 2014; 306:e945-964; PMID: 24549398

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.

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 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 *PPAR α* . 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 re-esterification to prevent depletion of the intracellular triglyceride pool.

Transcriptomic studies often identify expressed genes that have yet to be 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	<i>Elovl3</i>	fatty acid biosynthetic process ²⁷	<i>Zic1</i>	regulation of transcription, DNA-templated ²⁸				
2	<i>Ucp1</i>	oxidative phosphorylation uncoupler activity ²⁹	<i>Ucp1</i>	oxidative phosphorylation uncoupler activity ²⁹				
3	<i>Slc27a2</i>	long-chain fatty acid-CoA ligase activity ³⁰	<i>Cpn2</i>	enzyme regulator activity ³¹				
4	<i>Fabp3</i>	long-chain fatty acid transporter activity ³²	<i>Fabp3</i>	long-chain fatty acid transporter activity ³²				
5	<i>S100b</i>	calcium-dependent protein binding ³³	<i>Slc27a2</i>	long-chain fatty acid-CoA ligase activity ³⁰				
6	<i>Acot11</i>	fatty acid metabolic process ³⁴	<i>9130214F15Rik</i>	—				
7	<i>Cpt1b</i>	carnitine O-palmitoyltransferase activity ³⁵	<i>Kng1</i>	negative regulation of peptidase activity ³⁶				
8	<i>Cox7a1</i>	oxidation-reduction process ³⁷	<i>Ppara</i>	sequence-specific DNA binding transcription factor activity ³⁸				
9	<i>Gyk</i>	glycerol-3-phosphate biosynthetic process ³⁹	<i>Pank1</i>	coenzyme A biosynthetic process ⁴⁰				
10	<i>Kng1</i>	negative regulation of peptidase activity ³⁶	<i>Cpt1b</i>	carnitine O-palmitoyltransferase activity ³⁵				
11	<i>Otop1</i>	Anti-inflammatory activity ⁴¹	<i>Me3</i>	oxidation-reduction process ⁴²				
12	<i>PPARα</i>	sequence-specific DNA binding transcription factor activity ³⁸	<i>Plet1os (2310014F07Rik)</i>	Non-coding RNA highly expressed in BAT, heart and skeletal muscle				
13	<i>Cpn2</i>	enzyme regulator activity ³¹	<i>Cox7a1</i>	oxidation-reduction process ³⁷				
14	<i>Pdk4</i>	regulation of fatty acid biosynthetic process ⁷	<i>Gnas</i>	G-protein β /gamma-subunit complex binding ⁹				
15	<i>Cyp2b10</i>	oxidation-reduction process	<i>Gmpr</i>	oxidation-reduction process ⁴³				
16	<i>Dio2</i>	thyroid hormone metabolic process ⁴⁴	<i>Myo5b</i>	regulation of protein localization ⁴⁵				
17	<i>Pank1</i>	coenzyme A biosynthetic process ⁴⁰	<i>Acot11</i>	fatty acid metabolic process ³⁴				
18	<i>Naglt1a (AI317395)</i>	sodium-dependent glucose transporter (predicted)	<i>Mapt</i>	regulation of microtubule-based movement				
19	<i>Fbp2</i>	gluconeogenesis ⁴⁶	<i>Dio2</i>	thyroid hormone metabolic process ⁴⁴				
20	<i>Aspg</i>	lipid catabolic process ⁴⁷	<i>S100b</i>	calcium-dependent protein binding ³³				
21	<i>Esrrg</i>	sequence-specific DNA binding transcription factor activity ⁴⁸	<i>Ntrk3</i>	transmembrane receptor protein tyrosine kinase signaling pathway ⁴⁹				
22	<i>ldh3a</i>	oxidation-reduction process ⁵⁰	<i>Esrrg</i>	sequence-specific DNA binding transcription factor activity ⁴⁸				
23	<i>Slc25a20</i>	Mitochondrial substrate/solute carrier ⁵¹	<i>Fbp2</i>	gluconeogenesis ⁴⁶				
24	<i>4931406C07Rik</i>	ester hydrolase activity	<i>Tspan18</i>	—				
25	<i>Cidea</i>	Lipid metabolic process ¹³	<i>Otop1</i>	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 (Nagl1a). 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 Nagl1a 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 study⁴ and that of the Wolfrum group⁶ 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 gene⁹ 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 adipocyte¹⁰ 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-1 α , Plin5, PPAR α , 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-1 α , IL-6, IGF-1, BMP8B, and prostaglandins (reviewed in¹²) 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 in¹⁴). NGF is however also produced by cultured brown adipocytes¹⁵ 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 NH₂-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 cancer¹⁸ 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 as 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

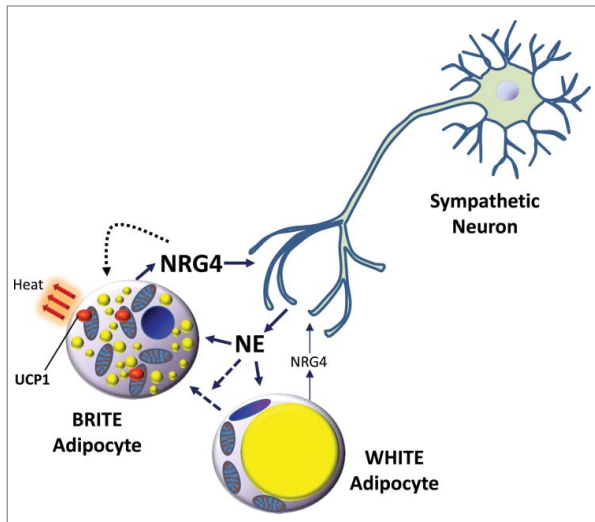


Figure 1. NRG4 is brown adipocyte adipokine that promotes neurite outgrowth. Neuregulin 4 (Nrg4) is more highly expressed in brown adipocytes compared to white adipocytes. Upon cold exposure, norepinephrine (NE) is secreted and activates brown fat as well as initiating the “browning” of white fat resulting 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-1 α 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 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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