Molecular and Cellular Endocrinology 276 (2007) 24–35

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Characterization of Tusc5, an adipocyte gene co-expressed in peripheral neurons☆

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Received 28 February 2007; accepted 22 June 2007

Abstract

Tumor suppressor candidate 5 (Tusc5, also termed brain endothelial cell derived gene-1 or BEC-1), a CD225 domain-containing, cold-repressed gene identified during brown adipose tissue (BAT) transcriptome analyses was found to be robustly-expressed in mouse white adipose tissue (WAT) and BAT, with similarly high expression in human adipocytes. Tusc5 mRNA was markedly increased from trace levels in pre-adipocytes to significant levels in developing 3T3-L1 adipocytes, coincident with several mature adipocyte markers (phosphoenolpyruvate carboxykinase 1, GLUT4, adipsin, leptin). The Tusc5 transcript levels were increased by the peroxisome proliferator activated receptor-γ (PPARγ) agonist GW1929 (1 μg/mL, 18 h) by >10-fold (pre-adipocytes) to ∼1.5-fold (mature adipocytes) versus controls ($p < 0.0001$). Taken together, these results suggest an important role for Tusc5 in maturing adipocytes. Intriguingly, we discovered robust co-expression of the gene in peripheral nerves (primary somatosensory neurons). In light of the marked repression of the gene observed after cold exposure, these findings may point to participation of Tusc5 in shared adipose–nervous system functions linking environmental cues, CNS signals, and WAT–BAT physiology. Characterization of such links is important for clarifying the molecular basis for adipocyte proliferation and could have implications for understanding the biology of metabolic disease-related neuropathies.
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Keywords: Adipogenesis; Brain endothelial cell derived gene-1 (BEC-1); PPARgamma; Neuropathy; Thermogenesis

1. Introduction

The regulation of body weight and energy storage involves cross-talk among many physiological systems which respond to alterations in hormones, tissue metabolites, environmental cues, and nutrients. Experimental systems which display significant metabolic malleability and coincident large shifts in gene expression, protein translation, and metabolite flux are especially helpful in discovering new molecular players involved with this cross-talk, since the signal-to-noise ratio for detection of biochemical and molecular changes is high. For instance, the impressive thermogenic capacity of rodent brown adipose tissue (BAT) may be leveraged to identify metabolically-relevant genes and to reveal new insights into subcellular metabolite pathways which are relevant body-wide. Spiegelman and colleagues identified peroxisome proliferator-activated receptor-γ (PPARγ) co-activator-1 (PGC-1α) as a cold-induced protein in brown adipocytes (Puigserver et al., 1998; Wu et al., 1999), and subsequent work established an important role for this protein in modulating expression of metabolism-related genes in non-BAT tissues including liver and muscle (see Puigserver, 2005). Based on the premise that genes relevant to thermogenesis and metabolism display temperature-sensitive expression changes

☆ This research has been previously presented in part: P. Oort, C. Farber, C. Warden, J.F. Medrano, L. Pennacchio, S. Adams. Characterization of an Obesity Candidate Gene: Unique Co-Expression in Adipocytes and Sensory Nervous System Structures. Obesity 14(9) Supplement: A205 (Abstract 645-P). Annual Mtg. of the North American Assoc. for the Study of Obesity (NAASO), October 2006, Boston, MA, USA.

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0303-7207/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.mce.2007.06.005

in BAT, brown fat transcriptomes from mice kept at thermoneutrality (33 °C, 3 week), just below thermoneutrality (22 °C), or maintained in the cold (4 °C, up to 2 days) have been compared (Adams et al., 2001; Yu et al., 2001; Yu et al., 2002). The unique mitochondrial carrier protein CGI-69 (Yu et al., 2001) and a novel acyl-CoA thioesterase (Adams et al., 2001) were among the many differentially-expressed genes induced by cold exposure and whose tissue expression patterns suggested activities beyond brown fat. Furthermore, BAT expression patterns of a large suite of metabolically-important genes – coupled to *in vivo* measures of BAT metabolite flux – revealed that cold elicits a coordinated, paradoxical and energetically futile co-induction of *de novo* lipid synthesis and mitochondrial ß-oxidation in this tissue (Yu et al., 2002). It was thus inferred that distinct subcellular pools of lipogenic versus inhibitory malonyl-CoA exist within brown adipocytes (malonyl-CoA inhibits carnitine palmitoyltransferase-1 and hence ß-oxidation, but also serves as an important lipogenic metabolite)(Yu et al., 2002).

Relatively little is known about warmth-induced or cold-repressed transcripts in BAT or white adipose tissue (WAT). Genes which normally function in "governor" pathways for non-shivering thermogenesis and/or BAT growth are hypothesized to be down-regulated with exposure to cold. Cold-repressed genes might also participate in activities important to temperature-related shifts in macronutrient utilization and storage. Herein, we characterize a cold-repressed transcript discovered in the course of previously-described murine BAT studies (see Adams et al., 2001; Yu et al., 2001; Yu et al., 2002 for animal model details) and initially termed adipose abundant protein (AAP) (WO/2002/097036, Adams 2002). Three splice variants of the human AAP orthologue were cloned from human tissue libraries with transcript lengths of 1.5, 1.9, and 2.7 kb, each containing an identical open reading frame (ORF) encoding a 177 amino acid, 19.1 kDa protein containing two carboxy-terminus transmembrane regions (WO/2002/097036, Adams 2002). The AAP gene has subsequently been termed tumor suppressor candidate 5 (Tusc5) in the public databases (GenBank: NM_177709 and NM_172367 for murine and human sequences, respectively), a nomenclature derived from a report suggesting loss of the gene in certain lung cancer cells (Konishi et al., 2003). Initial studies led to the concept that this gene has a role in regulating BAT and white adipose tissue metabolic function or abundance (WO/2002/097036, Adams 2002): First, tissue expression patterns revealed relatively high Tusc5/AAP mRNA abundance in human WAT and hibernoma (an adipose growth with BAT characteristics) and in the WAT and BAT of mice, with a marked 81% reduction of WAT transcript levels in cold-exposed mice. Second, limited studies in a 3T3-L1 cell culture model indicated that the gene is induced during progression of adipocyte differentiation. Very recently, Shibata et al., identified rat Tusc5 (termed brain endothelial cell derived gene-1, or BEC-1, in their paper) as a gene robustly-expressed in WAT and BAT and, similar to our studies in the mouse, found that the gene was cold-repressed in BAT (Shibata et al., 2007).

The studies herein were designed to further understand the basic biology of Tusc5/BEC-1 and the timecourse of its induction during adipogenesis. To this end, we provide a comprehensive analysis of human and mouse Tusc5 tissue specificity, protein domain analysis, and we detail induction of the gene during the adipocyte differentiation program relative to multiple functional and growth-dependent adipocyte genes. In addition, we evaluated the hypothesis that Tusc5 expression, like several other genes important to adipocyte maturation and function is regulated by PPARγ. Marked Tusc5 mRNA up-regulation in differentiating 3T3-L1 adipocytes in parallel with several late adipogenesis markers and increases in expression following PPARγ agonist treatment are consistent with the idea that Tusc5 plays a role in the physiology of relatively mature adipocytes. The unexpected discovery that Tusc5 is co-expressed in peripheral neurons and WAT highlights a potential shared role for the protein in these systems, with implications for understanding molecular phenomena associated with neuropathies common in obesity and type 2 diabetes.

2. Materials and methods

2.1. 3T3-L1 adipocyte differentiation and PPARγ agonist studies

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) grown to between five and nine passages were induced to differentiate into adipocytes using a protocol similar to Farmer et al. (i.e., Stephens et al., 1999). Briefly, 2-day-confluent cells grown in high-glucose (25 mM) DMEM and 10% FBS (37 °C, 5% CO_2) in 6-well rat tail collagen-coated plates were exposed to differentiation medium containing 10 μg/mL bovine insulin (∼1.6 μM), 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 2 days. Cells were grown thereafter in maintenance medium (DMEM/10% FBS + 2.5 μg/mL insulin). Cell culture media was replaced daily. Proof-of-principle studies tested the ability of a single dose of the potent non-thiazoladinedione PPARγ agonist GW1929 (Brown et al., 1999) to stimulate Tusc5 gene expression in non-differentiated confluent 3T3-L1 cells or at various points in the adipocyte differentiation and maturation process as indicated. For these experiments, cells were cultured for the times indicated in media containing vehicle (DMSO; 0.1% by volume) or GW1929 (1 μg/mL, 2.02 μM; 0.1% by volume). At the timepoints indicated, RNA was prepared from 3T3-L1 adipocytes using Trizol-based methods for cell culture samples (Ambion, Austin, TX). RNA abundance and integrity were checked using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 bioanalyzer (Agilent, Foster City, CA) per manufacturer's instructions. Changes in glucose and lactate concentrations in aliquots from the culture media during the adipocyte differentiation timecourse studies (see Section 3) were measured using a YSI 2300 glucose-lactate analyzer (Yellow Springs, OH), and net uptake or output was calculated from metabolite levels pre- and post-treatment.

2.2. Gene expression analyses

RNA abundance of Tusc5 and other genes of interest was measured using quantitative real-time PCR. These assays utilized gene-specific TaqMan primers and 5′FAM-3′TAM labeled probes (Assays-on-Demand®, Applied Biosystems Inc., Foster City, CA; see Supplemental Tables 1 and 2) and were run in duplicate or triplicate for each sample using an ABI 7900HT instrument. For tissue panel gene expression patterns, one-step RT-PCR using total RNA as template was used. Human samples were assayed in 30 μL reactions (96-well format) containing: 100 ng RNA, 1× Master Mix (ABI One-Step RT-PCR Master Mix, part #4309169), 1× primer/probe mixture. Cycle conditions were 48 °C 30 min, 95 °C 10 min, then 45 cycles of 95 °C for 15 s/60 °C for 1 min. Mouse tissue panel samples were assayed in 12 μL reactions (384-well format) containing 50 ng RNA and 1× each Master Mix and primer/probe mix. Gene expression for all other studies was analyzed using a two-step process with preparation of cDNA from total RNA, followed by quantitative real-time PCR. Briefly, for each sample, cDNA was prepared from 5 μg of total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) followed by RNAse-H treat-