The transcription factor GATA2 regulates differentiation of brown adipocytes

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Brown adipose tissue (BAT) is a specialized mammalian tissue and a site of adaptive thermogenesis. Although the metabolic functions of brown and white adipocytes are distinct, terminal differentiation of both adipocyte lineages is regulated by well-characterized common transcription factors. However, the early stages of adipocyte differentiation and regulation of precursor cells are not well understood. We report here that GATA2 is expressed in brown adipocyte precursors, and its expression is downregulated in a differentiation-dependent manner. Constitutive expression of GATA2 suppressed expression of BAT-specific genes in brown adipocytes, whereas disruption of a GATA2 allele in brown preadipocytes resulted in significantly elevated differentiation and expression of several markers of brown adipogenesis. Collectively, these results show that GATA2 functions to suppress brown adipocyte differentiation, whereas reduction of GATA2 promotes brown adipogenesis.

Keywords: differentiation; transcription; brown adipocyte; obesity; metabolic regulation

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INTRODUCTION

Brown adipose tissue (BAT) is a specialized mammalian tissue involved in thermoregulation. In this tissue, thermogenesis occurs through the activity of uncoupling protein 1 (UCP1), an inner mitochondrial membrane protein that functions as a proton leak to deregulate ATP synthesis. Morphologically, brown adipocytes store triglycerides as multilocular droplets dispersed throughout

the cell amidst numerous mitochondria with dense cristae. BAT hypertrophies in response to prolonged exposure to cold (Bukowiecki *et al*, 1986) or adrenergic stimulation (Geloën *et al*, 1992), and deteriorates in mass and function in obese animals (Himms-Hagen, 1989; Nisoli *et al*, 2000).

The sequence of events that regulate formation of BAT during development is poorly understood. Although BAT and white adipose tissue (WAT) are functionally distinct, they share transcriptional regulators that mediate their terminal differentiation (Rosen & Spiegelman, 2000). Early in the differentiation programme, C/EBP β and C/EBP δ are transiently expressed (Cao *et al*, 1991; Yeh *et al*, 1995) and contribute to the expression of PPAR γ (Wu *et al*, 1995; Darlington *et al*, 1998), an indispensable activator of terminal adipocyte differentiation (Rosen *et al*, 1999), and C/EBP α , another important transcriptional component of adipogenesis (Rosen *et al*, 1999; Wu *et al*, 1999b). PPAR γ and C/EBP α operate synergistically to promote the expression of genes found in both adipocyte lineages, such as aP2 fatty-acid-binding protein, fatty acid synthase (FAS), leptin and adiponectin (Rosen & Spiegelman, 2000). Whereas PPAR γ can induce differentiation of C/EBP α ^{−/−} cells, PPAR γ ^{−/−} cells lack the ability to differentiate (Rosen *et al*, 1999). Conversely, C/EBP α ^{−/−} cells can be induced to differentiate with PPAR γ ; however, they cannot form functionally intact adipocytes, especially with regard to insulin action and glucose metabolism (Wu *et al*, 1999b). Compared with white adipocytes, mature brown adipocytes preferentially express UCP1 and PPAR γ -coactivator 1 (PGC1 α ; Ross *et al*, 1992; Puigserver *et al*, 1998). PGC1 α is regulated by adrenergic stimulation in brown adipocytes, and transactivates genes associated with mitochondrial biogenesis, respiration and thermogenesis (Puigserver *et al*, 1998; Wu *et al*, 1999a).

Recent studies have indicated a role for the GATA family of transcription factors in white adipogenesis (Tong *et al*, 2000, 2005; Menghini *et al*, 2005). In this study, we investigated the function of GATA proteins during brown adipocyte differentiation. Our findings indicate that constitutive expression of GATA2 blocked transcriptional activation and expression of genes specific to the brown lineage as well as some, but not all, common adipogenic molecules. Reduction of GATA2 expression through

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conditional inactivation of a *GATA2* allele in brown preadipocytes resulted in increased expression of several BAT-specific and mitochondrial genes. Taken together, these observations show that *GATA2* has a crucial role in brown adipocyte differentiation.

RESULTS AND DISCUSSION

Our laboratory has previously described the role for two members of the *GATA* transcription factor family in white adipocyte differentiation. Unlike WAT, which expressed both *GATA2* and *GATA3*, BAT expressed only *GATA2* and not *GATA3* (Fig 1A). The level of *GATA2* expression in BAT is lower than other genes examined in WAT and BAT such as *UCP1*, *aP2* and *PPAR γ* (Fig 1A). As further controls for gene expression in WAT and BAT, we also measured ATP synthase, cytochrome *c* oxidase subunits IV and II (*COX IV* and *COX II*) and *PGC1 α* (1.1, 2.6-, 5.8- and 22-fold higher in BAT compared with WAT, respectively; Fig 1A). The expression pattern of *GATA* factors in BAT was mimicked in brown adipocyte cell lines, in which *GATA2* but not *GATA3* expression was detectable (Fig 1B). The highest expression levels of *GATA2* gene transcripts were observed in brown preadipocytes before differentiation, and rapidly diminished after the initiation of adipogenesis (Fig 1B). Proper differentiation of this cell line over a time course was verified by expression of adipogenic genes common to both white and brown cell types, such as *aP2* and *PPAR γ* , as well as induction of brown adipocyte genes such as *UCP1*, *PGC1 α* or *COX IV* (Fig 1B). *C/EBP α* expression was also induced by day 2 (5.8-fold) and maintained afterwards, whereas ATP synthase expression was slightly induced (1.5-fold) and remained steady throughout differentiation (not shown). The differentiation-dependent expression pattern of *GATA2* can be independently replicated in both the HB2 (Irie *et al*, 1999) and FVB (Klein *et al*, 1999) brown adipocyte cell lines (data not shown). *GATA2* protein was also detectable in HIB-1B preadipocytes and was suppressed after differentiation of these cells into adipocytes (Fig 1C).

The decrease in *GATA2* is concomitant with an increase in adipogenic gene expression, which suggests that downregulation of *GATA2* may be crucial for attaining the mature brown adipocyte phenotype. To test this, we examined the impact of constitutive expression of *GATA2* on brown adipocyte differentiation. Retroviral transduction was used to constitutively express *GATA2* or the empty vector as controls in several brown adipocyte cell lines (Fig 2A). The expression levels of exogenous *GATA2* messenger RNA and protein were confirmed by reverse transcription-PCR (RT-PCR) and western blotting (Fig 2A; data not shown). Constitutive expression of *GATA2* strongly suppressed genes associated with terminally differentiated brown adipocytes such as *UCP1*, *PGC1 α* and *COX IV* (Fig 2B). The expression of *COX II* was also strongly suppressed (fourfold), but regulation of ATP synthase β -subunit was mild (1.5-fold), as examined by real-time RT-PCR during differentiation of these cells. Although there was also suppression of *PPAR γ* and *aP2* mRNAs, other adipogenic genes such as *C/EBP α* and *adipsin* were expressed at similar levels in control and *GATA2*-expressing cells (Fig 2B), which suggests differential regulation of brown versus common adipogenic genes by *GATA2*.

To determine whether *GATA2* has the ability to regulate these genes differentially, we examined the effect of *GATA2* on the transcriptional activities of the *C/EBP α* , *UCP1* and *PGC1 α*

Fig 1 | *GATA* factors in murine brown adipocytes and adipose tissue. (A) Expression of *GATA2*, *GATA3* and other brown (*UCP1* and *PGC1 α*) or common (*PPAR γ* and *aP2*) adipocyte markers was examined in total RNA samples isolated from interscapular brown adipose tissue (BAT) and epididymal white adipose tissue (WAT), by northern blot analysis. (B) Expression of *GATA2* during brown adipocyte differentiation (induced at day 0). *GATA2*, *GATA3*, *UCP1*, *PGC1 α* , *PPAR γ* , *aP2* and *COX IV* (cytochrome *c* oxidase subunit IV) gene expression was examined by northern blot analysis in HIB-1B cells collected at 2-day intervals during differentiation. Cells are stimulated with isoproterenol for 6 h before collection at each time point. *GATA2* and *GATA3* panels are exposed overnight, others for 6 h. (C) *GATA2* protein in HIB-1B cells in preadipocytes (pre-Ad) and differentiated (4-day) adipocytes (Ad). *EtBr*, ethidium bromide.

promoters, which contain putative *GATA*-binding sites, in HIB-1B preadipocytes. Interestingly, *GATA2* had no effect on the transcriptional activity of the *C/EBP α* promoter (Fig 2C). In contrast, the basal activity of both *UCP1* (a 2.9 kb 5'-flanking region) and *PGC1 α* (a 230 bp 5'-flanking region) promoters was suppressed two- to threefold by *GATA2* (Fig 2D,E). These data indicate that *GATA2* is not a general suppressor of all promoters expressed in these cells. These results also indicate that *GATA2*

Fig 2 | Regulation of brown adipogenesis by GATA. (A) Levels of GATA2 messenger RNA and GATA2 protein were examined to confirm the exogenous expression through retroviral transduction of HIB-1B cells. (B) Effect of GATA expression on brown adipogenesis. Gene expression patterns in GATA-expressing (at day 6) or vector-expressing (at days 0 and 6) HIB-1B cells after stimulation with isoproterenol for 6 h before collection. Ethidium bromide (EtBr) staining was included to reflect loading and integrity of RNA. (C–E) Relative luciferase activities of 1.6 kb C/EBP α , 2.9 kb UCP1 and 230 bp PGC1 α promoters in the presence or absence of GATA2 in HIB-1B preadipocytes. UCP1 promoter activity was measured in the presence of exogenous PGC1 for activation. (F) Coexpression of PGC1 α (P α) and GATA2 in HIB-1B cells. Cells were differentiated and total RNA was isolated for analysis of PPAR γ and aP2 gene expression.

may act directly on the UCP1 promoter, or that the effect on UCP1 is secondary to the suppression of PGC1 α . To address the latter, we tested whether GATA2-mediated suppression of UCP1 expression could be rescued by exogenous PGC1 α . Ectopic expression of PGC1 α in GATA2-expressing cells failed to restore suppression of gene expression by constitutive GATA2 expression, most strikingly for UCP1 (Fig 2F). As before, the impact on suppression of PPAR γ and aP2 was modest, but these, too, were not enhanced by coexpression of PGC1 α with GATA (Fig 2F). Therefore, it is likely that GATA2 regulates UCP1 and perhaps other brown adipocyte target genes independent of the suppression of PGC1 α . Alternatively, it is also possible that GATA2 interacts directly with PGC1 α or another protein of functional significance, to alter either its activity or access to its transcriptional targets. For example, GATA proteins have been shown to bind directly to C/EBP transcription factors and interfere with their activity in white adipocytes (Tong *et al*, 2005). Thus, it will be of interest to examine whether such interactions also exist for other molecules that are crucial in the differentiation or function of brown and white adipocytes

such as PGC1 α . Finally, much higher levels of exogenous PGC1 α might be necessary to reverse the effects of GATA in brown adipocytes.

Interestingly, constitutive GATA expression had virtually no effect on the expression of the transcriptional regulator C/EBP α (Fig 2B), and also did not influence the transcriptional activity of a 1.6 kb C/EBP α promoter (Fig 2D). This suggests a biased regulation of brown adipocyte genes compared with those associated with general adipocyte differentiation. Consistent with this, overexpression of GATA2 did not significantly affect the lipid accumulation that is typically associated with adipocyte maturation (Fig 3A). Ectopic expression of GATA3 also resulted in a similar profile, with little impact on adipocyte morphology (Fig 3A). This might be the cumulative result of intact C/EBP α expression and/or residual PPAR γ action in these cells. Conversely, the structural integrity of mitochondria was compromised in GATA2-expressing cells, as reflected by the decreased density of cristae shown by electron microscopy (Fig 3B). Taken together, our results are consistent with the ability of GATA2, which is native to the

Fig 3 | Adipocyte morphology in GATA-expressing cells. (A) Lipid accumulation in HIB-1B cells stably expressing GATA2 or GATA3 shown by oil red O staining after differentiation for 6 days. (B) Electron microscopic evaluation of mitochondria in HIB-1B cells constitutively expressing GATA2. HIB-1B cells expressing GATA2 or empty vector were differentiated for 6 days, pelleted and processed for electron microscopy. Arrows indicate mitochondria in representative cell populations.

brown adipocyte, to selectively block features of brown adipocyte differentiation.

A *GATA2*^{-/-} embryonic stem (ES) cell-culture system is not feasible for the study of brown adipocyte differentiation because of the difficulty of obtaining a consistent and stable phenotype representing brown adipocytes (J.T. & G.S.H., unpublished observations). Thus, to examine whether the progression of brown adipogenesis could be altered by the loss of GATA2 in preadipocytes, we generated a brown adipocyte cell line using primary brown adipocytes from animals that harbour one conditional *loxB* allele of *GATA2* (*G2LoxB*; Fig 4A; Chang *et al*, 2002). Subsequently, we exogenously expressed Cre recombinase to induce a targeted deletion of the *GATA2* allele in these cells (Δ GATA2) and examined their ability to differentiate and support brown adipocyte gene expression profiles. As shown in Fig 4B,C, GATA2 mRNA and protein expressions were significantly reduced in Δ GATA2 cells, indicating the efficacy of the experimental procedure. In comparison with their control counterparts, Δ GATA2 brown adipocytes with reduced GATA2 expressed significantly higher levels of BAT-specific genes after differentiation, such as UCP1 and PGC1 α (Fig 4D). Reduction of GATA2 gene expression also resulted in increased expression of

Fig 4 | Impact of reduced GATA2 expression in brown adipogenesis. (A) Conditional targeting of *GATA2* gene in G2LoxB cells examined by Southern blot analysis. Disruption of *GATA2* locus (Δ GATA2) after the treatment of G2LoxB heterozygous cells with Cre recombinase, along with untreated G2LoxB (–) and wild-type (WT) controls for comparison. (B) Expression levels of *GATA2* mRNA in Δ GATA2 cells compared with Cre-untreated (–) and WT controls, examined by northern blot analysis. EtBr, ethidium bromide. (C) *GATA2* protein levels in Δ GATA2 cells compared with Cre-untreated (–) controls. *GATA2* protein was examined by western blotting with an anti-mouse *GATA2* antibody. (D) Cre-treated G2LoxB preadipocytes (Δ G) and untreated controls (C) were differentiated and stimulated with 1 mM dibutyryl-cAMP and then examined for various brown adipocyte markers. (E) Expression of genes that encode mitochondrial proteins and PPAR γ in Cre-treated G2LoxB preadipocytes (Δ G) and untreated controls (C). ATP syn β , ATP synthase β . (F) Lipid accumulation in differentiated (day 6) Δ GATA2 adipocytes after staining with oil red O.

mitochondrial genes such as COX IV, COX II and ATP synthase (Fig 4E). There was a 1.8-fold increase in PPAR γ expression in Δ GATA2 cells compared with controls. These conditions also mildly enhanced cellular capacity for differentiation, as indicated by greater intracellular lipid accumulation (Fig 4F). We also attempted to determine whether GATA2 has a role in the formation of BAT in chimeric mice produced by GATA2 $^{-/-}$ ES cells. Analysis of tissues from these chimeric animals indicated that GATA2 $^{-/-}$ cells were not excluded from BAT or WAT (not shown). Although the chimaera analysis should be interpreted cautiously because of the limitations of that system, together with our cell-based experiments, these data indicate that GATA2 is not required for white or brown adipogenesis; however, its reduced expression in precursor cells potentiates brown adipocyte differentiation.

Several lines of evidence have indicated an important role for BAT in metabolic homeostasis (Himms-Hagen, 1989; Lowell et al, 1993; Guerra et al, 2001). Mechanisms that regulate the formation and function of this tissue may also contribute to overall energy balance (Kopecky et al, 1995; Guerra et al, 2001). Hence, one possible function of GATA2 in brown adipocytes may be to modulate brown adipocyte differentiation in response to the changing metabolic demands of the organism. It will be of interest to investigate further whether GATA serves as a metabolic 'fulcrum', in which it activates energy dissipation versus storage by gating brown and white adipocyte differentiation. Other transcription factors have also been shown to function in such a capacity. Of particular note are FOXC2, SRC1, TIF2, PTEN and RIP140, all of which have significant roles in mediating energy balance and fuel metabolism at the molecular level through transcriptional regulation (Cederberg et al, 2001; Picard et al, 2002; Wang et al, 2003; Komazawa et al, 2004; Leonardsson et al, 2004). The possible interaction of GATA proteins with these molecules has yet to be fully determined. In addition, its early expression in preadipocytes suggests a possible role in cell-lineage determination, as has been described for Retinoblastoma (Hansen et al, 2004). *In vivo* manipulation of GATA2 expression, specifically in the preadipocytes of adipose tissue, will further our understanding of this potential role.

METHODS

Cell culture. HIB-1B and HB2 cells were cultured as described previously (Ross et al, 1992; Irie et al, 1999), with minor modifications (see the supplementary information online). Primary cells were isolated from transgenic animals as described previously (Irie et al, 1999).

RNA isolation and analysis, western blots. Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) and then analysed either by northern blot analysis or real-time RT-PCR, as described previously (Maeda et al, 2005). To study the expression, both endogenous and exogenous GATA2 mRNA expression primers common to human and mouse sequences were used. GATA2 antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA) and Cell Signaling (Beverly, MA, USA) and 200 µg total cellular protein extract was used to examine GATA2 expression.

Viral transfections. For retroviral transduction, stable transfectants were generated as described previously (Xu et al, 1999), with

minor modifications. To generate Δ GATA2 cell lines, immortal GATA2 $^{lox/+}$ p53 $^{-/-}$ brown adipocytes obtained from the above procedure were infected with the AdCMV-Cre adenovirus (provided by Randy Johnson, UCSD, San Diego, CA, USA), as described in the supplementary information online.

Luciferase assays. The luciferase reporter constructs directed by C/EBP α , PGC1 α and UCP1 5'-flanking sequences were provided by M. Daniel Lane (Johns Hopkins University, Baltimore, MD, USA), Marc Montminy (Salk Institute for Biological Studies, La Jolla, CA, USA) and Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, LA, USA). For experimental details, see the supplementary information online.

Oil red O staining. Differentiated adipocytes were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS), stained with 0.5% (w/v) oil red O stain (Sigma, St Louis, MO, USA) and visualized by standard light microscopy at $\times 40$ magnification.

Electron microscopy. Differentiated HIB-1B cells expressing either GATA2 or vector control were trypsinized, pelleted at 1,500 r.p.m. for 5 min, washed with PBS at pH 7.0 and pelleted again at 1,500 r.p.m. for 5 min. Cells were processed for electron microscopy as described previously (Stearns et al, 2001), with minor modifications (see the supplementary information online).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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