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SHORT COMMUNICATION Investigating the involvement of the ATF6 α pathway of the unfolded protein response in adipogenesis

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The unfolded protein response (UPR) is activated by endoplasmic reticulum stress resulting from an accumulation of unfolded or mis-folded proteins. The UPR is divided into three arms, involving the activation of ATF-6, PERK and IRE-1, that together act to restrict new protein synthesis and increase the production of chaperones. Recent studies have implicated the PERK and IRE-1 components of the UPR in adipocyte differentiation. In this study, we investigate the importance of ATF6 α during adipogenesis using stable knockdown of this protein in the model adipogenic cell line, C3H10T1/2. Reduction of ATF6 α expression by >70% resulted in impaired expression of key adipogenic genes and reduced lipid accumulation following the induction of adipogenesis. In contrast, loss of ATF6 α did not impair the ability of cells to undergo osteogenic differentiation. Overall, our data indicate that all three arms of the UPR, including ATF6 α , must be intact to permit adipogenesis to occur.

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INTRODUCTION

Adipose tissue dysfunction is proposed to contribute significantly to obesity-related metabolic disease.¹ Consequently, understanding the molecular basis of adipogenesis may provide therapeutic approaches to improve adipose function and the pathophysiology of obesity. Adipose tissue is not only the predominant site of lipid storage but also functions as an important nutrient sensing and endocrine organ.² The endoplasmic reticulum (ER) is a major site for protein and lipid synthesis, and in states of overnutrition elevated levels of ER stress have been reported.³ The unfolded protein response (UPR) to cellular stress is activated by the accumulation of unfolded proteins in the ER. The UPR involves three distinct pathways initiated by the activation of the ER membrane kinase PERK, the non-conventional mRNA splicing enzyme IRE-1 and the transcription factor ATF6a. These pathways work together to re-establish cellular homeostasis. Broadly, the PERK and IRE-1 pathways reduce the translation of new proteins, whereas the IRE-1 and ATF6 α pathways increase the capacity of the ER to process the accumulated unfolded proteins.⁴ The importance of the UPR has been demonstrated in highly secretory cells, such as pancreatic β cells⁵ and plasma B cells, which exhibit elevated levels of the UPR and in B-cells this is essential for terminal differentiation.⁶ Several studies link ER stress with the pathophysiology of obesity.^{7,8} However, given the high secretory capacity and metabolic activity of adipocytes, the involvement of the UPR in normal adipocyte development has also recently been investigated. This has revealed that a physiological level of the UPR may be required for adipogenesis to occur. Specifically, PERKdeficient mouse embryonic fibroblasts and 3T3-L1 preadipocyte cells display reduced adipogenic capacity, reduced expression of lipogenic genes and impaired lipid accumulation.⁹ A detailed study of the IRE-1 arm of the UPR revealed that this too has an important role in adipogenesis. Expression of mRNA encoding an inactive form of the transcription factor XBP-1 (XBP-1u) is induced by C/EBP β during early adipogenesis. Upon activation of the UPR, XBP-1u mRNA undergoes non-conventional splicing by IRE-1 to generate an mRNA encoding the active XBP-1 protein (XBP-1s). In turn XBP-1s binds to and activates the promoter of the critical adipogenic transcription factor C/EBPα. Thus, loss of either XBP-1 or IRE-1 in mouse embryonic fibroblasts or 3T3-L1s results in impaired adipogenesis.¹⁰

ATF6 α is a transmembrane protein that, following activation of the UPR, undergoes proteolytic cleavage by site-specific proteases to produce a free 50 kDa active transcription factor, which upregulates the expression of ER chaperones.⁴ As both the PERK and IRE-1 arms of the UPR have key roles in adipocyte differentiation, we investigated whether this third arm, involving ATF6 α , is also required for adipogenesis.

RESULTS AND DISCUSSION

To determine the relative expression of ATF6 α in adipose tissue, the levels of ATF6 α mRNA were determined in a murine tissue panel (Figure 1a). As might be expected given the ubiquitous nature of the UPR, this revealed detectable levels of ATF6a mRNA in all tissues tested, including abundant expression in mesenteric, subcutaneous and brown adipose tissue. We next examined ATF6a mRNA expression in C3H10T1/2 mesenchymal stem cells following induction of adipogenesis using medium containing fetal bovine serum supplemented with insulin, dexamethasone and IBMX (IDM), as described previously.¹¹ ATF6 α mRNA was detected at all time points but did not significantly change as adipogenesis proceeded (Figure 1b). We also determined the expression of ATF6 α (p90) protein during adipogenesis in these cells. This revealed a modest increase in ATF6 α during the first 5 days of adipogenesis (Figure 1c), which subsequently decreased to undetectable levels at D8 (Figure 1c). The available antibodies were unable to detect the 50 kDa active form of ATF6 α (p50). Thus, we are unable to specifically analyse the proteolytic activation of ATF6 α during adipogenesis. However, these data demonstrate

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that ATF6 α protein is expressed in these cells and regulated during adipogenesis.

To examine the importance of ATF6 α in adipogenesis, we used shRNA to stably inhibit ATF6 α expression in C3H10T1/2 cells. Briefly, two shRNA sequences targeting $ATF6\alpha$ (shATF6 α -1; exon 1-2; 5'-AAGACTGGGAGTCGACGTTGT-3' and shATF6α-2; exon 4-5; 5'-AACTCAGCACGTTCCTGAGGA-3') were designed and cloned into RNAi-ready pSIREN-RetroQ vector (Clontech, Mountain View, CA, USA). Retroviruses were produced by transfecting ATF6 α shRNA constructs or a control shRNA vector into BOSC-HEK293 packaging cells. These were used to infect C3H10T1/2 cells, as previously described,¹¹ generating two ATF6a knockdown cell lines. Analysis of the shATF6 α expressing cell lines indicated ATF6 α mRNA expression was reduced by more than 70% in both cell lines when compared with the control cells across a time course of adipogenesis (Figure 2a). A second isoform of ATF6, ATF6B is highly homologous to $ATF6\alpha$ with a proposed inhibitory role on ATF6 α targets.¹² We therefore tested whether the ATF6 α shRNA affected ATF6 β mRNA expression. No significant difference was seen in ATF6 β expression levels in cells expressing either ATF6 α shRNA when compared with control cells, indicating that both shRNA are specific to ATF6 α (Figure 2b). Western blot analysis verified that ATF6 α protein levels, were also inhibited by shATF6 α -1 and shATF6 α -2, respectively, in D0 cells (Figure 2c).

We next examined the effect of ATF6a knockdown of the expression of key adipogenic genes during differentiation. Following induction of adipogenesis with IDM medium, control and $ATF6\alpha$ knockdown cell lines displayed similar increases in the expression of the early adipogenic transcription factor, C/EBPB (Figure 2d). However, the degree of induction was modestly, but significantly, reduced by ATF6 α knockdown. The induction of the adipogenic transcription factor PPARy was modestly, but significantly, reduced in ATF6 α knockdown cells at early time points up to day 3 and more markedly impaired as adipogenesis proceeded (Figure 2e). In addition, expression of the lipogenic transcription factor SREBP1c, the insulin-sensitive glucose transporter, GLUT4 and fatty acid-binding protein, aP2, all exhibited dramatically reduced levels of mRNA expression in the ATF6a knockdown cells (Figures 2f-h). Western blotting of aP2 expression demonstrated that this was not induced following adipogenic treatment of ATF6a knockdown cells, consistent with the mRNA expression data (Figure 2i). Overall, these data demonstrate that, at least in this cellular model of adipogenesis, ATF6 α expression is required for the normal induction of adipogenic gene expression.



Figure 1. ATF6 α tissue distribution and expression in developing adipocytes. (a) ATF6 α expression was determined by real-time PCR in a panel of murine tissues. Data are normalised to 18s RNA. Data are expressed as the means ± s.e.m. (b) ATF6 α expression was determined by real-time PCR in C3H10T1/2 cells grown to confluence then induced to undergo adipogenesis. Total RNA was collected at day 0, 1, 2, 3, 5 and 8 of differentiation and extracted using the RNEasy kit (Qiagen, Crawley, UK). Data are normalised to Cyclophilin A and are expressed as the means ± s.e.m. (n = 3). * Indicates significant difference from 0 h, P < 0.05 by ANOVA with *post hoc* Tukey's test. (c) Protein lysates were collected from C3H10T1/2 cells induced to differentiate for 0, 1, 2, 3, 5 or 8 days using RIPA buffer containing protease and phosphatase inhibitors. Samples were guantified using NIH Image J software (http://rsbweb.nih.gov/ij/) and ATF6 α intensity normalised to calnexin, n = 3, ± s.e.m., * indicates significance of P < 0.05 with respect to levels in undifferentiate cells. Representative western blots of ATF6, the adipocyte marker aP2 and the loading control calnexin (ab13504, Abcam) are shown from the same lysate samples.



Consistent with the impaired expression of these genes lipid accumulation, as measured by Oil red O staining,¹³ was markedly reduced in the ATF6 α -deficient cells (Figure 2j, upper panels). To ensure that this did not reflect a non-specific defect in cells lacking ATF6 α , these cells were also treated with osteogenic induction medium. After 5 days of differentiation alkaline phosphatase activity, a marker of osteogenesis, was visualised with BCIP/NBT. This revealed that the osteogenic capacity of these

cells was not impaired by ATF6 α knockdown (Figure 2j, lower panels). Thus, ATF6 α appears selectively important for adipocyte differentiation.

In conclusion, we have demonstrated that knockdown of ATF6 α significantly, and selectively, impairs the ability of C3H10T1/2 cells to differentiate into mature adipocytes. Further study is required to determine the precise mechanisms involved, and to determine whether this is also the case in other cellular and *in vivo* models of



Figure 2. Knockdown of ATF6 α expression inhibits adipogenesis. C3H10T1/2 cells stably expressing control shRNA (black bars), shATF6 α -1 (dark grey bars) or shATF6 α -2 (light grey bars) were induced to differentiate for 8 days. Total RNA collected at day 0, 1, 2, 3, 5 and 8 and extracted using the RNEasy kit (Qiagen). mRNA expression of (a) ATF6 α , (b) ATF6 β , (d) C/EBP β (e) PPAR γ , (f) SREBP1c, (g) GLUT4 and (h) aP2 were determined using real-time PCR. Data are normalised to Cyclophilin A and are expressed as means ± s.e.m. (n = 3). * Indicates significant difference in expression from control shRNA at the same timepoint, P < 0.05 by ANOVA with *post hoc* Tukey's test. Inserts show expanded view of early timepoints. (c) Protein lysates were collected from confluent control and ATF6 α knockdown cells and western blotted for ATF6 α and calnexin as described in Figure 1. (i) Protein lysates were collected from control and ATF6 α knockdown cells induced to differentiate for 0, 2, 3, 5 or 8 days, western blotted and aP2 expression quantified as described in Figure 1, n = 4, ± s.e.m., * indicates significance of P < 0.05 with respect to levels at the same time point in control cells. Representative western blots of aP2 and the loading control calnexin (ab13504, Abcam) are shown from the same lysate samples. (j) Control and ATF6 α deficient C3H10T1/2 cells were differentiated in IDM medium for 8 days and lipid accumulation assessed by oil-red O staining, as previously described¹³ (upper panels). Alternatively, cells were induced to undergo osteogenesis by treatment with Dulbecco's modified Eagle's medium supplemented with poly FBS, 10 mM Glycerol-2-phosphate, 50 µg ml⁻¹ ascorbic acid, 300 µg ml⁻¹ BMP2 and 1 µM dexamethasone (lower panels). Alkaline phosphatase activity, indicating osteogenic conversion, was visualised using BCIP/NBT (B1911, Sigma-Aldrich, St Louis, MO, USA).

adipocyte development. However, coupled to the previous studies of PERK and IRE-1, these data suggest that all three arms of the UPR must be intact to permit adipogenesis to occur.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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