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ORIGINAL ARTICLE

Cytoglobin: a potential marker for adipogenic differentiation in preadipocytes in vitro

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Abstract Obesity, mainly characterized by the excess fat storage, is a global health problem resulting in serious morbidity and mortality. Identification of molecular mechanisms in adipogenic differentiation pathway might lead to development of new strategies for diagnosis, prevention and therapy of obesity and associated diseases. Discovery of new genes and proteins in the differentiation pathway could help to understand the key specific regulators of the adipogenesis. Cytoglobin (Cygb), identified as a new globin family member protein, is expressed in various tissues. Although its interaction with oxygen and nitric oxide indicates the potential role in antioxidant pathways, the exact role remains unclear. In the current study, expression level of Cygb was determined in proliferating and differentiating 3T3-F442A cells by gene expression and protein expression analysis. Results revealed that Cygb expression up-regulated in differentiated cells in parallel with adipogenic differentiation markers; PPARγ, CEBPα and FABP4 expressions. Besides, Cygb overexpression in preadipocytes contributed to the adipogenic differentiation as verified by detection of higher lipid droplets and increased PPARγ, CEBPα and FABP4 expressions with respect to control cells. These findings will shed light on the unknown roles of Cygb in adipogenesis and obesity.

Keywords Cytoglobin · Obesity · 3T3-F442A · Preadipocyte · Adipogenic differentiation

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Introduction

Cytoglobin (Cygb) as a hexacoordinated protein has been discovered as the fourth member of the globin family in 2002 by Burmester and his collegues (Burmester et al. 2002). Cygb was discovered in hepatic stellate cells (HSCs) in 2001 and identified as stellate cell activation-associated protein (STAP) (Kawada et al. 2001) and was renamed as cytoglobin in 2002. Cygb expression was detected in many types of vertebrate tissues (Burmester et al. 2002). Expression of Cygb in the fibroblasts of rat kidney, spleen and pancreas has been reported and claimed to display potential roles in fibrogenesis and production of extracellular proteins such as collagen (Nakatani et al. 2004; Hankeln et al. 2004). Similarly, Cygb has

been proven to be involved in activation of HSC and liver fibrogenesis (Tateaki et al. 2004). Hypoxia induced expression of Cygb in the brain, eye, liver, heart and muscle tissues of mice has been reported, indicating the potential protective role against hypoxia (Fordel et al. 2007). Expression of Cygb in the retina might be an explanation to oxygen binding and transport during hypoxic conditions (Ostojic et al. 2006). Cygb could serve an O_2 scavenging and supplying function, and could contribute to the detoxification process of reactive oxygen species (Fordel et al. 2004). Because Cygb has specific functions during hypoxia, its expression in hypoxic tumor environment is important for tumor progression. Cygb might act as a tumor suppressor gene and downregulation of Cygb has been observed in human malignancies (Oleksiewicz et al. 2013). Although exact role of this protein is unknown, nuclear localization in many tissues leads the hypothesis of interaction with other proteins to regulate gene transcription for several cellular processes (Geuens et al. 2003).

Obesity has gained a great interest in recent years as a major global health problem due to the high prevalence (Nguyen and El-Serag 2010). Obesity and consequences are associated with several diseases including diabetes, cardiovascular disorders, cholesterol and kidney problems (Christiaens et al. 2012). Obesity is mainly the result of expanded adipose tissue which is important for production of various cytokines and controls metabolic conditions in the body. In parallel with the obesity incidence and comprehensive research on adipocyte biology, burgeoning interest in regulation of adipogenesis at the gene level has emerged. In order to understand obesity progression, adipogenesis and lipid storage mechanism, transcriptional regulators controlling the process should be completely identified. Peroxisome proliferator-activated receptor g (PPARγ) and CCAAT/enhancer binding protein (CEBPα) regulate the adipogenic transformation as key transcriptional factors. PPARγ and CEBPα induced growth arrest is the critical step for fully differentiated phenotype of preadipocytes (Rosen et al. 2000). Although these two transcription factors have critical roles in the adipogenesis, other additional genes such as Wnt, Foxo1, and SREBP-1 have also been reported to have important roles in adipogenesis (Oishi et al. 2005).

Extensive research for the identification of transcriptional regulatory pathways of adipogenesis and new marker proteins are required to solve obesity mechanism. Expression of Cygb has not been shown in adipose tissue before and the role in adipogenesis remains unclear. In the present study, Cygb expression profile in the differentiated preadipocytes was evaluated using a commonly used preadipocyte, 3T3-F442A cells, and Cybg-modified preadipocytes were examined in terms of adipogenic differentiation potential.

Materials and methods

Cell culture and adipogenic differentiation

3T3-F442A (EF300; Kerafast, Boston, MA, USA) preadipocytes were used in adipogenic differentiation experiments. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life technologies Inc., Carlsbad, CA, USA) containing 10% fetal calf serum (FCS, Life technologies Inc.) and 1% penicillin–streptomycin–amphotericin (PSA, Life technologies Inc.). Cells were passaged before reaching confluency during culture to maintain differentiation properties. Cell differentiation was performed using a modified protocol according to the previously published one (Ayala-Sumuano et al. 2011). Briefly, 7×10^4 3T3-F442A cells were seeded onto 6-well plates (Corning, Corning, NY, USA) and incubated with DMEM containing 10% FCS and 1% PSA for 2 days to obtain confluency. The medium was renewed and the cells were incubated extra two days. After post-confluency, cells were treated with DMEM containing 10% fetal bovine serum (FBS, Life technologies Inc.), 1% PSA and 5 μg/mL insulin (Sigma, St. Louis, MO, USA) for 8 days (Fig. 1). The differentiation medium was refreshed every other day for 8 days. The same differentiation procedure was applied to the 3T3-F442A–GFP and 3T3-F442A–Cygb cells.

Viral transduction of 3T3-F442A cells

The vector plasmid containing the sequence corresponding to the 228-bp Cygb was ligated into pLenti-III-2A-GFP (abm, Richmond, CA, USA, Fig. 5a). Lentiviral vector stocks for GFP and Cygb expressing vectors were prepared by calcium phosphate transfection of 293T cells (ATCC, CRL-3216; Manassas, VA, USA) using pLenti-III-Cygb-2A-GFP or pLenti-III-

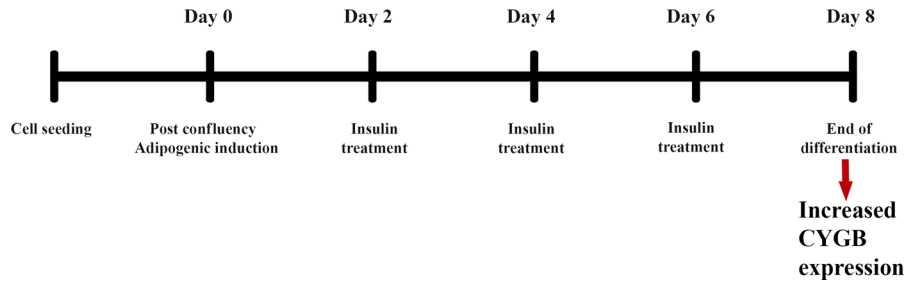

Fig. 1 Diagram showing the differentiation procedure of 3T3-F442A cells. Cygb expression increased at the end of the adipogenesis process

2A-GFP, pCMVDR8.2DVPR (Addgene, Cambridge, MA, USA) and pMD2.G (VSVG, Addgene). Viral supernatants were harvested for 3 days, filtered through 0.45 μm filter and stored at −80 °C until use.

Transduction of 3T3-F442A cell with viral supernatants of GFP and Cygb was performed in the presence of 8 μg/mL of polybrene and fresh media at 1:1 ratio for 24 h. Transduced cells were selected with 2 μg/mL puromycin treatment for 10 days. Cygb overexpression in transduced cells was confirmed by RT-PCR and western blot analysis.

Oil red staining

Differentiated 3T3-F442A–GFP and 3T3-F442A–Cygb cells were stained with Oil red O (ORO) (Sigma, St. Louis, MO, USA) to observe lipid vesicles. Briefly, ORO stock solution (340 mg ORO in 100 mL isopropanol) was diluted in distilled water (3 parts oil red:2 parts dH_2O). Fixed cells were treated with 60% isopropanol for 5 min at room temperature and followed by ORO staining for 10 min at room temperature. Cells were washed with dH_2O and visualized by an inverted microscope (Zeiss PrimoVert, Göttingen, Germany) equipped with an AxioCCam ERc5s camera and Zen 2011 software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Isopropanol extracted ORO absorbance was measured at 490 nm by an ELISA plate reader (Biotek, Winooski, VT, USA).

Gene expression analysis by RT-PCR

Total RNAs from differentiated cells at day 0, 2, 4 and 8 and differentiated 3T3-F442A–GFP and 3T3-F442A–Cygb cells were isolated using High Pure RNA-isolation kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. cDNAs were synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Optimized primers (QuantiTect Primer Assays; Qiagen, Valencia, CA, USA) for PPARγ, CEBPα, fatty acid binding protein 4 (FABP4) were used to confirm adipogenic differentiation. Primers for Cygb (F 5′ TCGGCCAAGCAGTACTTCAG 3′, R 5′ TGTCTGGGTCATGCAGGTTC 3′) and β-actin (F 5′ GACAGGATGCAGAAGGAG 3′, R 5′ TGATCCACATCTGCTGGA 3′) were designed by using Primer-BLAST online software from the National Center for Biotechnology (Bethesda, MD, USA). SYBR Green staining method was used to quantify relative mRNA levels of the genes of interest. All RT-PCR experiments were performed using an iCycler RT-PCR system (CFX Real Time System; Bio-Rad, Singapore).

Immunocytochemical analysis

Cells were fixed using 4% paraformaldehyde at day 0, 2, 4 and 8 of differentiation for 30 min at 4 °C, followed by permeabilization with 0.1% Triton-X 100/PBS solution for 5 min. Then, the cells were washed with phosphate buffer solution (PBS, Life technologies Inc.) and blocked with 1% bovine serum albumin (BSA, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were incubated with the following primary antibodies; anti-PPARγ (1:200 dilution; MA5-14889; Sigma), anti-CEBPα (1:400 dilution; 8178; Cell Signaling Technology, Beverly, MA, USA), anti-FABP4 (1:100 dilution; sc-271529; Santa Cruz Biotechnology), and anti-Cygb (1:200 dilution; sc-66855; Santa Cruz Biotechnology) overnight at 4 °C. Cells were then treated with AlexaFluor 488 FITC conjugated goat anti-rabbit or anti-mouse IgG (1:200 dilution; ThermoFisher, Waltham, MA, USA) secondary antibodies for 1 h at room temperature. Nuclei were stained with DAPI (0.5 μg/mL; Sigma), and samples were visualized with a confocal microscope (LSM 700; Zeiss, Heidelberg, Germany).

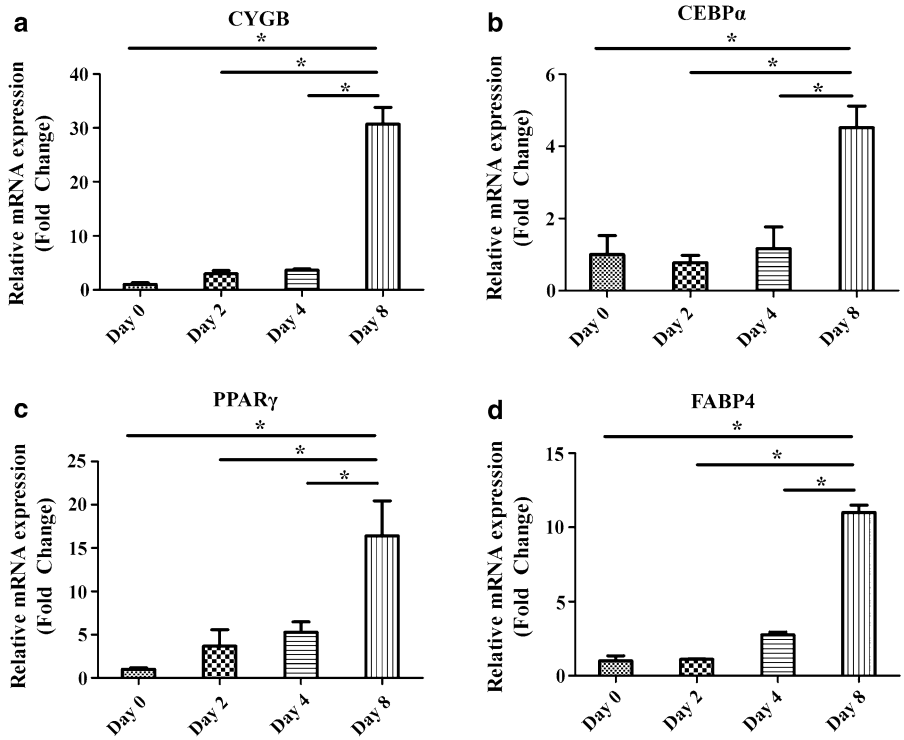

Fig. 2 Gene expression analysis of Cygb, PPARγ, CEBPα and FABP4 during differentiation process. Indicated genes showed an increased in relative mRNA expression in a time dependent manner. *$P < 0.05$. *Notes*: Results were analyzed by one-way ANOVA and Tukey's posttest

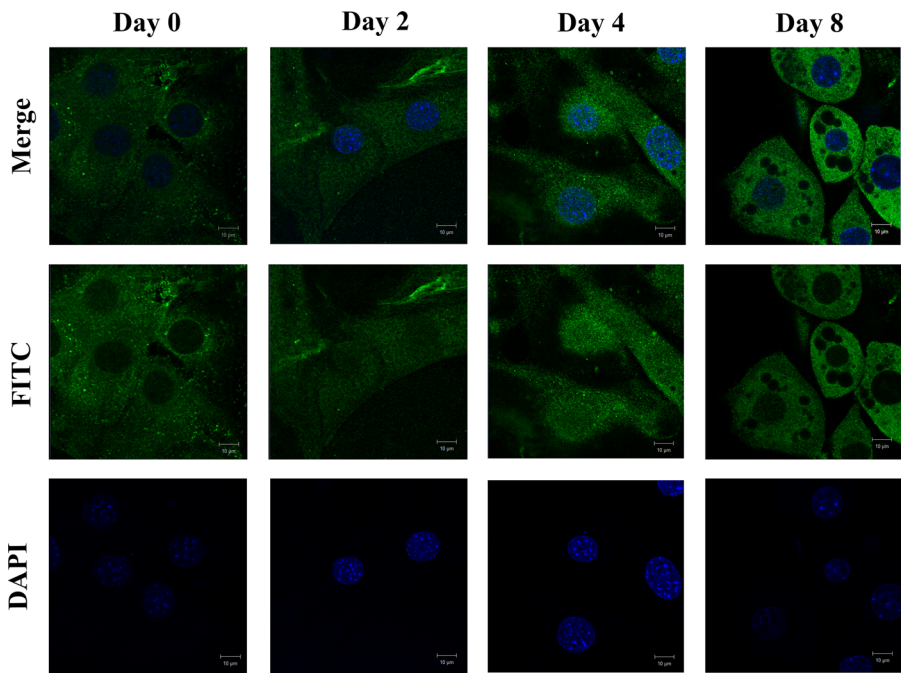

Fig. 3 Immunocytochemistry analysis of Cygb during differentiation process. Cygb expression increased during differentiation process. DAPI: Nuclei staining, FITC: Cygb staining. *Scale bar* 10 μm

Western blot analysis

All chemicals used in immunoblotting assays were purchased from Biorad Laboratories (Richmond, CA, USA). Primary antibodies against PPARγ (1:1000 dilution; MA5-14889; Sigma), CEBPα (1:1000 dilution; 8178; Cell Signaling Technology), FABP4 (1:1000 dilution; sc-271529; Santa Cruz Biotechnology), and Cygb (1:1000 dilution; sc-271529; Santa Cruz Biotechnology) were used for western blot analysis. Total protein was isolated from cells at day 0, 2, 4 and 8 of differentiation using RIPA Buffer (sc-24948, Santa Cruz Biotechnology) and protein concentrations were estimated using BCA assay (23227, Pierce, Rockford, IL, USA). Protein samples were loaded to Any kD™ Mini-PROTEAN® TGX™ precast gels (456-9033, Biorad, Hercules, CA, USA) at a concentration of 30 μg/lane. Then, proteins were transferred to nitrocellulose membranes (162-0115, Biorad, Munich, Germany). The membranes were incubated with blocking solution containing 5% non-fat dry milk (170-6404, Biorad). The membranes were incubated with the primary antibodies at 4 °C for 16 h. After washing with TBS-T three times, the membranes were incubated with anti-rabbit secondary antibody (sc-2004, dilution 1:5000, Santa Cruz Biotech) prepared in blocking buffer for 1 h at room temperature. β-Actin (1:5000 dilution, 3700S, Cell Signaling Technology) was used as an internal control and images were taken by using the luminometer system (Biorad). Relative band intensities were calculated using Image Lab software (Biorad) and normalized to the respective β-Actin band intensities. Results were represented as fold change of control.

Statistical analysis

The data were statistically analyzed using one-way analysis of variance and Tukey post hoc test. The values of $P < 0.05$ were considered statistically significant.

Results

Cygb gene expression increased throughout differentiation of 3T3-F442A cells

Cygb gene expression levels analyzed by RT-PCR increased in differentiated 3T3-F442A cells in a time-dependent manner and reached to a peak level at the end of day 8. Fully differentiated 3T3-F442A cells expressed high levels of Cygb as found for other adipogenesis related genes including PPARγ, CEBPα and FABP4 which also were upregulated at the end of 8 days. Cygb showed a similar gene expression pattern with PPARγ, CEBPα and FABP4. At the end of differentiation process, Cygb expression increased an approximately 30-fold with respect post-confluence cells (Fig. 2).

Cygb immunocytochemistry in differentiated cells

Enhanced Cygb protein expression was also confirmed by immunocytochemistry experiments. Remarkable

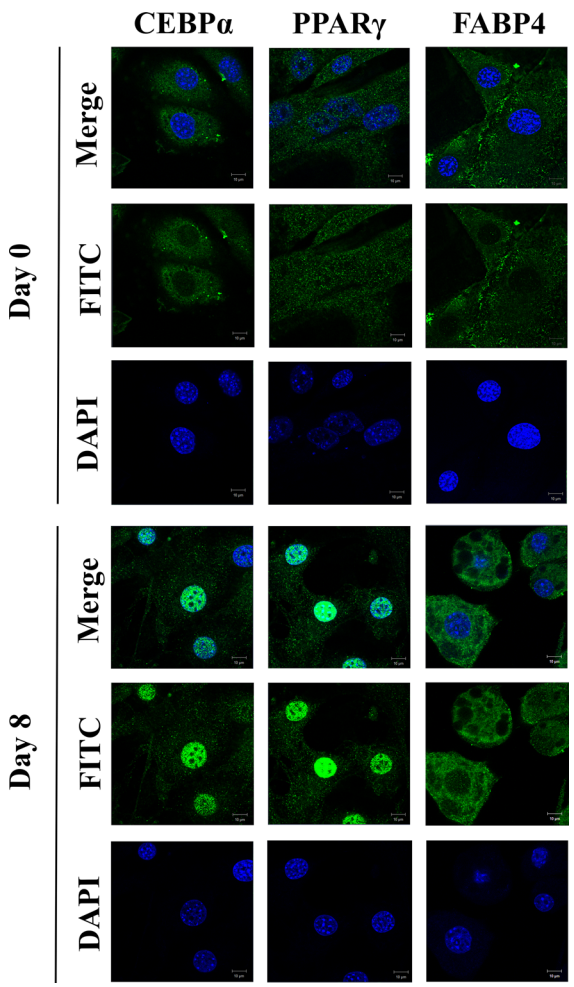

Fig. 4 Immunocytochemistry analysis of PPARγ, CEBPα and FABP4 during differentiation process. PPARγ, CEBPα and FABP4 staining at day 0 and day 8 is shown. Expression of PPARγ, CEBPα and FABP4 increased during differentiation process. DAPI: Nuclei staining, FITC: Cygb staining. *Scale bar* 10 μm

Cygb expression was detected in differentiating cells starting from day 0 to day 8. Expression levels increased in a time-dependent manner. Differentiated cells with a rounded morphology showed increased expression levels for Cygb (Fig. 3). While 3T3-F442A preadipocyte cells were differentiating to lipid accumulating mature adipocytes, PPARγ, CEBPα and FABP4 expression levels also increased, indicating the marker protein expressions in differentiating cells (Fig. 4).

Cygb expression increased at the protein level during differentiation

Cygb, PPARγ, CEBPα and FABP4 protein expression levels were detected by Western blot analysis. Cygb expression increased at the protein level in a time-dependent manner similar to RT-PCR and immunocytochemistry analysis. PPARγ, CEBPα and FABP4 protein expression levels were higher at day 8 compared to post-confluence cells, indicating the successful differentiation. In parallel with adipocyte marker expressions, higher levels of Cygb expression were observed at day 8 (Fig. 5a). An approximate 10-fold increase was noted for Cygb protein expression consistent with other marker protein expressions (Fig. 5b).

Cygb overexpression increased adipogenesis potential of 3T3-F442A cells

Cells were successfully transduced with viral vectors expressing Cygb or GFP (Fig. 6a). Cygb overexpression in 3T3-F442A preadipocyte cells were confirmed by RT-PCR (Fig. 6b). Although both transduced cells were differentiated into mature adipocytes and stored visible lipid droplets, Cygb overexpression was found to enhance lipid accumulation. Cygb overexpressing cells were accumulating more lipid droplets and relative oil red absorbance was high (Fig. 6c). Similar results were obtained for gene expression analysis of PPARγ, CEBPα and FABP4 expression. Gene expression analysis revealed that PPARγ, CEBPα and FABP4 expressions were significantly higher in 3T3-F442A–Cygb cells, proposing the contribution of Cygb in adipogenic differentiation and as a result of marker gene expression (Fig. 6d).

Discussion

Differentiation of precursor cells is regulated by several molecular events at the transcriptional level and characterized by several morphological changes. Understanding the development of various cell lineages including bone, muscle, neuron or endocrine cells is of great interest to solve regenerative medicine problems in clinics. Adipogenic precursor cells and adipogenesis are the interesting research fields as adipose tissue is regulating the energy metabolism, some of the endocrine hormones, and disturbance in the regulation would result in diabetes and obesity (Must et al. 1999).

A wealth of observation has been documented for obesity by using in vitro and in vivo adipocyte differentiation models. Identification of molecular

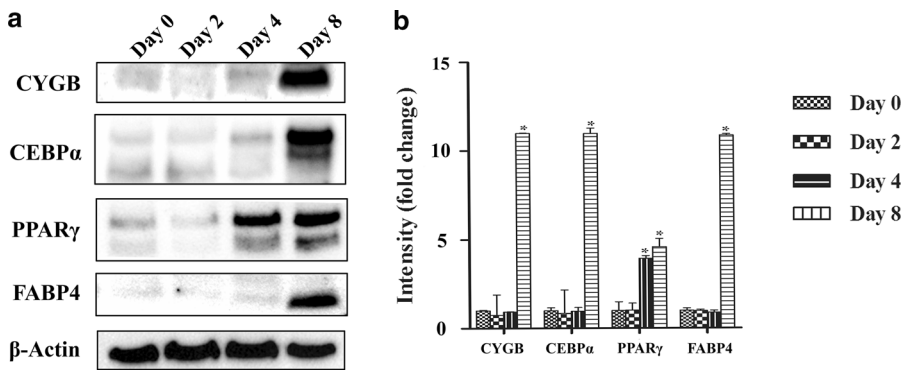

Fig. 5 Western blot analysis of Cygb, PPARγ, CEBPα and FABP4. during differentiation process. **a** Western blot results of Cygb, PPARγ, CEBPα and FABP4 during differentiation process, **b** quantitative results of western blot band intensities. *$P < 0.05$. *Notes*: Results were analyzed by one-way ANOVA and Tukey's posttest

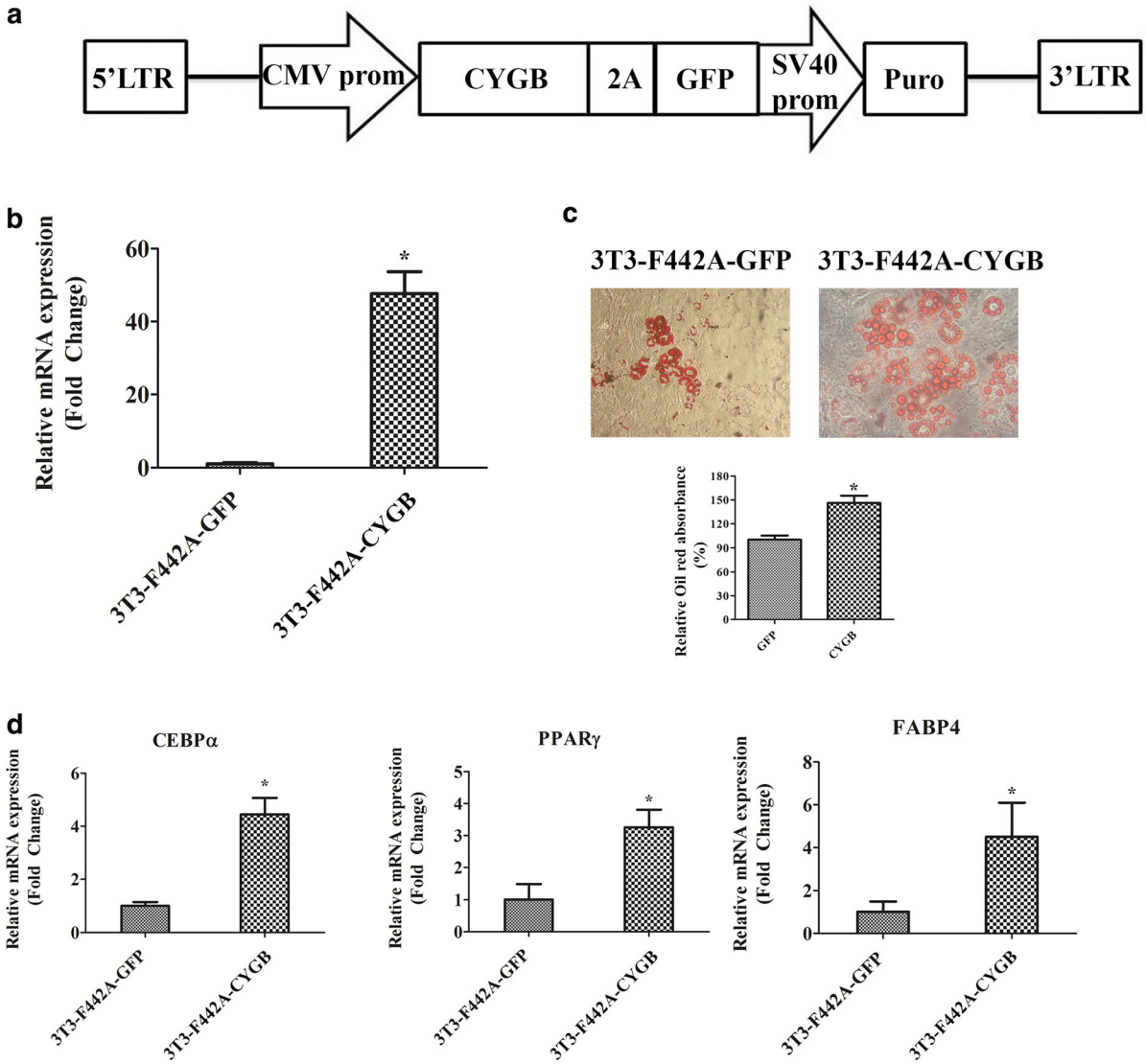

Fig. 6 Cygb overexpression increases adipogenic differentiation. **a** Structure of Cygb gene construct, **b** relative mRNA expression level of Cygb in transduced cells, **c** oil red staining of differentiated 3T3-F442A–GFP and 3T3-F442A–Cygb cells, **d** gene expression levels of PPARγ, CEBPα and FABP4 in differentiated 3T3-F442A–GFP and 3T3-F442A–Cygb cells at the end of 8 day differentiation period. $*P < 0.05$. *Notes*: Results were analyzed by one-way ANOVA and Tukey's posttest

mechanism in adipogenesis pathways could provide critical information for obesity. 3T3-F442A preadipocyte cell is a well-known model for in vitro adipogenesis. Cells enter to the additional rounds of cell division (mitotic clonal expansion) after post-confluency which is induced by the addition of differentiation cocktail to the cells. This process is controlled by transcription factors PPARγ and CEBPα at the molecular level. Expression of these two proteins induces a series of events for a fully differentiated adipocyte phenotype. These events include the binding to specific DNA reagents and acting as transcriptional activation of adipogenesis related genes. After these earliest events during adipogenesis, PPARγ and CEBPα activate the expression of some downstream mediators including glycerophosphate dehydrogenase (GPDH), glucose transporter type 4 (Glut4) and FABP4 which

characterize the mature adipocyte phenotype (Spiegelman et al. 1993). Identification of specific key genes might help to control fat storage and development of therapeutic strategies for obesity and metabolic disorders.

Cygb is a globin family member protein which is sensitive to hypoxia and oxidative stress and binds oxygen (O_2), carbon monoxide (CO) and nitric oxide (NO). It functions as an O_2 sensor and takes roles in protection from oxidative stress, cell proliferation, cancer progression and collagen synthesis (Burmester et al. 2007; Le Thi Thanh Thuy et al. 2016). Although some of the important roles of Cygb have been identified in recent years, the exact role in many cellular processes has not been known yet. Distribution of Cygb in different tissues and all organs might indicate important functions of this protein in different cellular processes. The upregulated expression of Cygb gene in differentiated preadipocytes (mature adipocytes) was observed in microarray analysis by our group in a recent study (data not shown). In addition, Kao et al. (2009) demonstrated the overexpression of Cygb in adipose and lipoma tissue derived mesenchymal stem cells using microarray analysis. Cygb expression levels were significantly higher in lipoma tissues along with the VNN1, SLC16A6 and COL11A1 genes. Although the main objective of the study was not to evaluate the role and expression profile of Cygb in adipose tissue and its potential roles were not discussed in the study, the obtained results have implied the potential significance of Cygb in adipose tissue. However, there were no reports in the literature explaining the role of Cygb in adipogenesis. Exact identification of Cygb's role in adipogenesis will clear our understanding of molecular pathways which are important for obesity and its progression in the obese population. Our findings would be the first study in the literature representing the Cygb's positive contribution to adipogenesis.

In the current study, we evaluated the expression levels of Cygb during differentiation of 3T3-F442A cells along with other adipocyte markers and important transcription factors. Cygb expression increased at gene and protein level in a time-dependent manner. The expression was significantly high at the end of 8 days, when preadipocytes become mature lipid storing adipocytes. Cygb expression profile demonstrated a similar pattern with important adipogenic transcription factors including PPARγ, CEBPα and adipogenic differentiation marker FABP4. Transcriptional activation of PPARγ enhances the FABP4 expression and leads the adipogenic differentiation and lipid accumulation in the preadipocytes (Rosen and MacDougald 2006). As Cygb expression upregulated at the end of differentiation similar to FABP4, Cygb might act as an adipogenic regulator throughout the differentiation procedure. This study revealed the upregulation of Cygb in mature adipocytes and showed a potential adipogenic differentiation marker for the first time. In order to confirm positive roles of Cygb in adipogenesis, we overexpressed the Cygb gene in 3T3-F442A preadipocytes and observed the adipogenic differentiation. Cygb overexpressing cells were found to store higher levels of lipid accumulation and PPARγ, CEBPα and FABP4 expression with respect to GFP expressing control cells. These results proposed that Cygb expression is important for adipogenesis and might be a critical indicator for obesity.

Although further studies should be completed to elucidate the exact role of Cygb in adipogenesis, this study might be a starting point for its role in obesity. The exact role of Cygb in the regulation of transcription factors PPARγ, CEBPα should be identified in detail in further studies. Identification of new marker genes for adipogenic differentiation and obesity would be a promising approach for developments in research and might lead to new discoveries for the clinics. The role of Cygb in adipogenesis and molecular pathway should be identified in further studies.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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