Adipose tissue deletion of Gpr116 impairs insulin sensitivity through modulation of adipose function

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A R T I C L E   I N   P R E S S

Article history:
Received 1 April 2012
Revised 1 August 2012
Accepted 4 August 2012
Available online xxx

Edited by Laszlo Nagy

Keywords:
G protein-coupled receptor 116 (GPR116)
Adipogenesis
3T3-L1 preadipocyte
Adipose tissue specific knockout
Insulin resistance
Lipodystrophy
Adiponectin

1. Introduction

Adipose tissue is traditionally regarded as an inert organ for storage of excess energy in the form of triacylglycerol (TG). However, this notion has been challenged by the findings during the past two decades. Adipose tissue is now well regarded as a highly complex and dynamic endocrine organ since it secretes a panel of lipid derivatives and bioactive peptides, termed ‘adipokines’, and thereby serves as a master regulator in maintaining systemic energy homeostasis [1]. Adipose dysfunction, characterized by an abnormal expression of adipokines [2] and the presence of a low-grade, chronic inflammation [3,4], are closely associated with abnormal expression of adipokines and the presence of a low-grade, chronic inflammation [3,4], are closely associated with obesity, type 2 diabetes and cardiovascular disease [5]. Increasing evidence suggests that adipose tissue dysfunction plays a prominent role in the development and progression of metabolic disorders including insulin resistance, type 2 diabetes and cardiovascular diseases [5].

Therefore, identification of key factors that control the physiology of adipose tissue would lead to new therapeutic approaches for prevention and treatment of metabolic related diseases.

G protein-coupled receptor 116 (GPR116) is a novel member of the G protein-coupled receptors and its function is largely unknown. To investigate the physiological function of GPR116 in vivo, we generated adipose tissue specific conditional Gpr116 knockout mice (CKO) and fed them on standard chow or high fat diets. Selective deletion of Gpr116 in adipose tissue caused a pronounced glucose intolerance and insulin resistance in mice, especially when challenged with a high fat diet. Biochemical analysis revealed a more severe hepatosteatosis in CKO mice. Additionally, we found that CKO mice showed a lowered concentration of circulating adiponectin and an increased level of serum resistin. Our study suggests that GPR116 may play a critical role in controlling adipocyte biology and systemic energy homeostasis.

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exert anti-inflammatory and insulin sensitizing actions [11]. The beneficial effects of ω-3 FA treatment were abrogated in GPR120 knockout mice. In addition, GPR120 promotes secretion of GLP-1 and increases circulating levels of insulin [12].

GPR116 belongs to the LNB-TM7 subfamily of GPCRs. This protein is known to be highly expressed in lung and can be processed into four fragments, namely, presequence, proEGF2 (α fragment), Ig repeats (β chain), and TM7 (γ chain) [13]. The natural ligand(s) and physiological role of GPR116 remain elusive. We found that GPR116 is also present in adipocyte cell lines and mouse adipose tissue, suggesting that this protein might be a modulator of adipose tissue function. Therefore, in the current study, we investigated the potential role of this protein in adipocytes using both in vitro and in vivo based approaches.

2. Materials and methods

2.1. Differentiation of 3T3-L1 adipocytes and oil red O staining

Mouse 3T3-L1 preadipocytes were normally cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Hyclone). Two days after reaching confluence (day 0), the cells were subjected to adipogenic differentiation in differentiation medium containing 5 μg/ml insulin (Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine (Calbiochem). Two days later the medium was replaced with DMEM supplemented with 10% FBS and 5 μg/ml insulin for additional two days before changed to the normal medium until day 8.

For oil red O staining, 3T3-L1 adipocytes were fixed in 10% formalin for 5 min, followed by incubation in fresh formalin for 1 h before washed briefly with 60% isopropanol and stained for 10 min in freshly prepared 60% oil red O solution. For quantitative analysis, oil red O was eluted with 100% isopropanol and its optical density was measured at 500 nm in a spectrophotometer (Beckman Coulter).

2.2. Isolation of adipocytes and stromal-vascular cells from adipose tissues

Isolation of mature adipocytes and stromal cells from fat tissue was performed as previously described [14]. Epididymal adipose tissue was dissected out, rinsed in phosphate-buffered saline (PBS), minced, and digested for 40 min at 37 °C in 0.1% (w/v) collagenase solution (Collagenase type I, dissolved in D-Hanks buffer). The digested tissue was filtered through a 250 μm nylon mesh to remove undigested tissues before centrifuged at 800 × g for 3 min. The floating layer (adipocytes) and the pellet (stromal cells) were collected and washed again in PBS.

2.3. siRNA transfection

SiRNA oligonucleotides were synthesized and purchased from Guangzhou Ribobio Company. Transfection of siRNA into the 3T3-L1 cells was performed with Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. The transfected cells were subjected to standard differentiation as described above.

2.4. Generation of adipose-specific Gpr116 knockout mice

Gpr116^{floxed/lox} mice and aP2 (Fabp4)-Cre transgenic mice were crossed to produce the adipose specific Gpr116 conditional knockout mice, Gpr116^{Gpr116^+/+} and aP2-Cre positive (Gpr116^{Gpr116^-/-}). Gpr116^{Gpr116^+/+} mice were used as the control. Primers used for Gpr116^{Gpr116^+/+} genotyping were: GCGACCACACGCTTGAAGAC as the forward primer (GF) and GTCCACCCTAACATTGC as the reverse primer (GR) while these for Cre genotyping are AGCGATG-GATTCCGTCTCTGG (CF) and AGCTTG CATGATCTCGGTATTTGAA (CR).

2.5. Total RNA extraction and quantitative PCR

Total RNA was isolated from cells and tissues using Trizol Reagent (Invitrogen). First-strand cDNA synthesis was performed with Superscript III Reverse Transcriptase (Invitrogen). Quantification of mRNA levels was performed using SYBR Premix Ex Taq™ (TaKaRa) under optimized conditions following the manufacturer’s instruction. The reference gene chosen was 18S ribosomal RNA. The primers were synthesized by Invitrogen in Shanghai, China. The sequences of the primers are listed in Supplementary Table 1.

2.6. Antibody production

Polyclonal antibody against GPR116 was raised in rabbits immunized with the peptide YKTDLERAFRAGYRT coupled with keyhole limpet haemocyanin.

2.7. Standard chow and high fat diet (HFD) feeding

Male mice [8-week-old, 129Sv (50%)/C57BL/6J (50%)] were housed in metabolism cages and maintained on a 12 h light–dark cycle at 23 °C and fed standard chow diet (SCD) (15.9 kJ/g, 10% of energy as fat, 20% of energy as protein, 70% of energy as carbohydrate; GB14924; Guangdong Medical Laboratory Animal Center, Guangzhou, China) or high fat diet (HFD) (21.9 kJ/g, 60% of energy as fat, 20% of energy as protein, 20% of energy as carbohydrate; D12492; Research Diet, New Brunswick, NJ, USA). Food intake was measured daily and body weight was measured weekly. All experiments with animals were approved by the Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

2.8. Glucose and insulin tolerance test

For glucose tolerance test, mice were fasted overnight and injected intraperitoneally (i.p.) with 20% glucose at a dose of 2 g/kg body weight. For insulin tolerance test, mice were starved for 6 h and i.p. injected with 0.5 U/kg body weight recombinant human insulin (Eli Lilly). Blood glucose was monitored from the tail vein blood using a glucometer (ACCU-CHEK Advantage; Roche Diagnostics, China, Shanghai, China) at various time points.

2.9. Analysis of plasma constituents

Blood samples were collected from cut tail tips of conscious overnight-fasted mice. Plasma triacylglycerol and cholesterol were measured using commercial kits (Triacylglycerol Kit and Cholesterol Kit; Proteomics analysis Jiancheng, Nanjing, China). Plasma adiponectin was measured by an ELISA kit (Millipore). Plasma resistin was measured by an ELISA kit (Raybiotech). Plasma levels of TNF-α and IL-1β were measured by ELISA kits (R&D).

2.10. Extraction and quantification of lipids

Tissues were homogenized in 2:1 chloroform/methanol solution in a 20:1 (20 ml/g) volume-to-weight ratio and incubated at room temperature for 6 h with gentle shaking. After that, H2SO4 (1 mM) was then added, centrifuged at the maximal speed for 15 min and the organic bottom layer was collected. The organic solvent was dried and the extracted lipids were re-dissolved in 50 μl ethanol. Triacylglycerol content was measured by the same Triacylglycerol Kit as mentioned above.
2.11. Histochemistry and immunohistochemistry

White adipose tissues were fixed in 4% formaldehyde overnight at room temperature immediately after the mice were sacrificed. Tissues were paraffinized and sectioned by microtome, and the slides were stained with hematoxilin and eosin (H&E) (Sigma) following the standard protocol. Sections were examined by light microscopy. Photomicrographs were scanned using an Abaton Scan 300/Color scanner. The digitized images were analyzed on a PC computer using the UTHSCSA ImageTool program (http://www.ddsdx.uthscsa.edu/dig/idtscd.html). Paraffin embedded sections of adipose tissues were stained with anti-rabbit F4/80 (1:100; Abcam; ab100790) to assess macrophage infiltration.

2.12. Statistical analysis

Data are expressed as means ± SEM. All comparisons were analyzed by unpaired, two-tailed Student’s t-test. A p value of p < 0.05 was considered significant.

3. Results

3.1. GPR116 is required for differentiation of 3T3-L1 preadipocytes

The mRNA of Gpr116 was detected in mouse adipose tissue by RT-PCR (data not shown). To further verify this finding, we isolated mature adipocytes and stromal vascular fraction from mouse white adipose tissue and the expression of Gpr116 in these two cell types was examined by qRT-PCR. The results demonstrated that Gpr116 mRNA was abundantly present in both mature adipocytes and stromal vascular fractions, at a level comparable to some of the known adipogenic markers such as peroxisome proliferator activator receptor-γ (PPARγ) and adipocyte fatty acid-binding protein (aP2/FABP4) (Fig. 1A). The expression profile of Gpr116 was also investigated in 3T3-L1 cells. Interestingly, we found that the expression level of Gpr116 was gradually and significantly increased during the first 6 days of differentiation but came back down at day 8 (Fig. 1B and C). This expression pattern is largely reminiscent to that of PPARγ, which plays a key role in adipocyte differentiation. The above mentioned findings suggest that GPR116 might play a potentially critical role in adipocyte physiology.

To further investigate the role of GPR116 in adipocyte differentiation, we reduced Gpr116 expression in 3T3-L1 cells by siRNA mediated knockdown (Fig. 1C). Compared to the cells transfected with scrambled siRNA, the efficiency of adipocyte differentiation was remarkably diminished in 3T3-L1 adipocytes with specific knockdown of Gpr116, as evidenced by a decreased level of PPARγ and aP2 (Fig. 1C). The impairment in adipocyte differentiation was further corroborated by oil red O staining and TG quantification (Fig. 1D and 1F). This result suggests that GPR116 is required for the in vitro adipogenesis of 3T3-L1 adipocytes. Since PPARγ was one of the most important regulators of preadipocyte differentiation, we wanted to determine whether up-regulation of PPARγ has any effect on the expression of Gpr116 mRNA. 3T3-L1 cells were treated by rosiglitazone, a known PPARγ agonist, during the process of adipocyte differentiation and the treatment increased Gpr116 mRNA levels further on day 6 and day 8 (Fig. 1H). In addition, transient transfection of pCMV driven human cDNA encoding GPR116 enhanced adipogenic differentiation in 3T3-L1 cells (Supplementary Fig. 1).

3.2. Generation of adipose tissue specific Gpr116 knockout mice

Next we sought to investigate the physiological role of GPR116 in adipose tissue in vivo. To this end, we generated adipose specific knockout mice of Gpr116 (CKO) as described in Session 2 (Fig. 2A). The Gpr116^lox/lox^ littermates were used as the wild type control (WT). The adipose selective deletion of Gpr116 was confirmed by Western blotting (Fig. 2B). Since aP2 is not only expressed in adipocytes but also in macrophages, we performed Western blotting analysis but did not detect any expression of Gpr116 in the peritoneal macrophages (data not shown).

3.3. Deletion of Gpr116 in adipose tissue exacerbates glucose intolerance and insulin resistance in mice

CKO and WT mice were born healthy and at expected Mendelian ratios (data not shown), the mice were fed on standard chow diet (SCD) or high fat diet (HFD) upon weaning and the metabolic related parameters were monitored. No significant differences were observed in body weight (Fig. 3A) and food intake (Fig. 3B) between WT and CKO mice, under both SCD and HFD feeding. However, glucose tolerance test (GTT) showed that deletion of Gpr116 in adipose tissue caused more severe glucose intolerance in mice under SCD for 8 wks (Fig. 3C) although the insulin sensitivity still remains the same as that in WT mice (Fig. 3E). Upon HFD challenge for 8 weeks, a more pronounced difference on glucose disposal ability was observed between WT and CKO mice. Specifically, ablation of Gpr116 in adipose tissue accelerated HFD induced glucose intolerance (Fig. 3D). Moreover, compared to the WT mice, CKO mice were more insulin resistant, as determined by the insulin tolerance test (ITT) (Fig. 3F). To evaluate the glucose stimulated insulin secretion, serum insulin levels were measured at various time points post glucose injection. We found serum insulin levels were lower in CKO mice at early time points compared to the WT mice on SCD and HFD (Fig. 3G and H), which may be responsible for the glucose intolerance observed in these mice.

Next, we examined the Akt phosphorylation in liver and skeletal soleus muscle upon insulin stimulation. Under standard chow conditions, there is no significant difference observed in Akt phosphorylation for these two tissues. However, Akt was hypophosphorylated in liver and muscle in CKO mice compared to the WT mice on HFD, indicating that insulin signaling was blunted in liver and muscle of CKO mice (Fig. 3I). Since increased gluconeogenesis in liver is one of the major contributors to glucose intolerance, the extent of liver gluconeogenesis was evaluated by determination of key genes involved using qRT-PCR. The results demonstrated that hepatic gluconeogenic genes G6pc and Pck1 were significantly upregulated in CKO mice on HFD feeding (Fig. 3J). Taken together, the data above indicated that mice with adipose tissue selective depletion of Gpr116 were more susceptible to high fat diet induced glucose intolerance and insulin resistance which is at least partially caused by a shift to the preference of hepatic glucose production.

3.4. GPR116 deficient mice exhibit more severe ectopic lipid accumulation and hyperlipidemia

In order to further define the physiological consequences from the deletion of Gpr116 in adipose tissue, phenotypes related to lipid metabolism were examined and compared in WT and GPR116 deficient mice. We performed oil red O staining to assess the extent of ectopic lipid accumulation in mice and found that the livers from CKO mice had a mildly elevated level of lipid accumulation under SCD (Fig. 4A, upper panel). The difference between WT and CKO mice was more pronounced in mice fed a HFD (Fig. 4A, lower panel). Like the observations in liver, skeletal soleus muscles from WT and CKO mice also had a comparable level of lipid deposition (Fig. 4B). The lipids in liver and skeletal muscle were also extracted and quantified by biochemical assays and the results mirrored the observations from the oil red O staining (Fig. 4C and D). The genes related to lipid metabolism were also examined in these two major metabolic tissues. As shown in Fig. 4E, the mRNA level of the genes
The lipid profiling in circulation was also examined in the WT and CKO mice. Data showed that plasma concentration of free fatty acid (Fig. 5A) and TG (Fig. 5B) was modestly higher in CKO mice upon HFD, whereas the serum cholesterol level was largely the same between the WT and CKO mice (Fig. 5C).

3.5. Deletion of Gpr116 leads to an alteration of adipocyte size and dysregulation of adipokine expression

Adipose tissue serves as a central commander in glucose and lipid metabolism of the body. To further delineate the molecular basis how GPR116 deficiency in adipose tissue causes an impaired

Fig. 1. GPR116 is involved in 3T3-L1 differentiation. (A) Stromal vascular cells and mature adipocytes were isolated from mice epidymal adipose tissue and the levels of Gpr116 mRNA were examined by qRT-PCR ($n = 4$). (B) Gpr116 mRNA was examined during differentiation of 3T3-L1 cells. (C) 3T3-L1 preadipocytes were transfected with scramble-siRNA or Gpr116 specific siRNA. QRT-PCR analysis of relative mRNA. (D) Oil red O staining of day 8 3T3-L1 cells was performed. (E) Western blotting analysis. (F) The measurement of triacylglycerol content was done on 24-well plates. (G) Western blotting analysis of GPR116 during differentiation of 3T3-L1 cells. (H) The levels of Gpr116 mRNA in 3T3-L1 preadipocytes treated with Rosiglitazone during differentiation.

insulin sensitivity and disturbed lipid homeostasis, we sought to identify the fundamental changes in adipose physiology per se. To this end, the epidymal fat pads were isolated and weighed. We did not observe significant difference in the weight of adipose tissue between the two groups (Fig. 6A). However, when the adipose tissue was subjected to H&E staining, we found the sizes of the adipocyte in the Gpr116 CKO mice were smaller compared with those in the WT mice (Fig. 6B and C). The reduction in adipose size was prominent under both SCD and HFD conditions. In addition, we found that adipocytes in CKO mice had a slightly decreased TUNEL staining compared with that in the WT mice (Supplementary Fig. 2).

The expression of several genes encoding key factors for adipogenesis and adipokines in mouse adipose tissue was also examined. Although most of the genes examined did not show much difference in expression, the mRNA level of Resistin was strikingly increased in GPR116 deficient adipose tissue under both SCD and HFD conditions (Fig. 6D). Moreover, the serum level of adiponectin and resistin was measured by ELISA and the results demonstrated that CKO mice exhibited a slight but statistically significant reduction of circulating adiponectin and increase of circulating resistin in comparison to the WT mice (Fig. 6E and F). Besides, there was no significant difference in terms of plasma leptin level between the control and CKO mice. Insulin resistance is also associated with inflammation that negatively impacts insulin sensitivity. While serum levels of TNF-α and IL-1β were similar between the WT and CKO mice on SCD, HFD elevated the secretion of these two proinflammatory cytokines in CKO mice than WT mice (Fig. 6G and H). As shown in Fig. 6I, expression of inflammatory genes such as IL-6, TNF-α, MCP-1 and IL-1β was increased in GPR116 deficient adipose tissues.

on HFD. Consistent with this result, CKO mice have increased F4/80 positive macrophages in adipose tissue on HFD (Fig. 6J).

4. Discussion

Key factors controlling the function of adipose tissue would represent potential drug targets for metabolic related diseases. Here we provide several lines of evidence, both in vitro and in vivo, demonstrating that GPR116 plays a critical role in adipose tissue function. First of all, the expression of Gpr116 was induced during differentiation of 3T3-L1 preadipocytes. Moreover, siRNA-mediated knockdown of Gpr116 largely inhibited the differentiation capacity of this cell line. On the other hand, mice with selective deletion of Gpr116 were more susceptible to high fat diet induced glucose intolerance and insulin resistance. Furthermore, these mice were featured by elevated circulating levels of TG, hetatostasis and smaller adipocytes.

Our in vitro study clearly showed that Gpr116 knockdown impaired 3T3-L1 preadipocyte differentiation. However, in case of...
mice, both the total body weight and the weight of the adipose tissue did not differ significantly between the WT and CKO mice. Results from in vitro and in vivo experiments seem inconsistent at first glance: knockdown of Gpr116 inhibited the differentiation of 3T3L1 preadipocytes; whereas in CKO mice, the body weight and the weight of the fat pad were not significantly reduced. However, the smaller adipocytes in CKO mice might be an indicator of impaired adipocyte differentiation, which, to certain extent, consistent with the in vitro results. Besides, aP2 is expressed relatively late in adipogenesis and mainly in mature adipocytes. Thus, the knockout mice mainly reflected the role of GPR116 in mature adipocytes.

On the other hand, these observations indicate that acceleration of aging and high fat diet induced insulin resistance observed in CKO was caused by a mechanism independent of obesity. Interestingly, similar case is also present in GPR120, another metabolic related protein in GPCR family. It was reported that downregulation of GPR120 resulted in inhibition of adipocytes differentiation [10] whereas GPR120 knockout mice had similar body weight with the WT mice [15]. It is possible that the unchanged body weight...
Therefore, activation of GPR116 might represent a potential alternative to adiponectin replacement therapy is technically challenging. It should also be noted that due to the high concentration of adiponectin in serum and the complex posttranslational modification, including assembly and secretion of various forms of adiponectin oligomers, involves a highly complex but organized machinery. Therefore, it is intriguing to investigate in the future whether GPR116 participates in the assembly and secretion of adiponectin. It should also be noted that due to the high concentration of adiponectin in serum and the complex posttranslational modification, the adiponectin replacement therapy is technically challenging. Therefore, activation of GPR116 might represent a potential alternative to adiponectin replacement therapy.

Taking together, in the current study, we investigated the role of GPR116 in adipose biology and systemic energy metabolism. GPR116 deficiency in adipocyte cell lines blocked the full differentiation of adipocytes, which is translated into dysfunctioning adipocytes in vivo, exemplified as smaller size, reduced adiponectin secretion and upregulated resistin. This study establishes GPR116 as a potential mediator linking adipose tissue dysfunction and systemic insulin resistance. GPR116 is an orphan receptor and the ligand for GPR116 is currently unknown. Fatty acids were found to be ligands for GPR116; however, the detailed mechanism is currently unclear.

Several phenotypic differences became more prominent in CKO mice when the mice were challenged with a high-fat diet. This phenomenon suggests that GPR116 might also act to protect the body from metabolic disorders under pathological conditions, while it is less involved in maintaining the normal function of the body. In addition, it is noteworthy that the most consistent changes observed in CKO mice, under both SCD and HFD, were from the adipose tissue, i.e., the smaller adipocyte size, higher resistin and lower adiponectin level. Hence the smaller adipocytes and abnormal expression of adipokines might represent the primary causes that in turn evoke the peripheral insulin resistance, hepatic steatosis, hyperlipidemia in CKO mice. It is possible that mice on SCD would exhibit similar phenotypes to those on HFD upon prolonged observation.

The circulating level of adiponectin was lowered in CKO mice. Nevertheless the mRNA level of adiponectin was at a similar level as the WT mice. Adiponectin is a pleotropic adipokine that possesses anti-inflammatory, anti-atherogenic and insulin-sensitizing effects [16]. The posttranscriptional maturation of adiponectin, including assembly and secretion of various forms of adiponectin oligomers, involves a highly complex but organized machinery. Therefore, it is intriguing to investigate in the future whether GPR116 participates in the assembly and secretion of adiponectin. It should also be noted that due to the high concentration of adiponectin in serum and the complex posttranslational modification, the adiponectin replacement therapy is technically challenging. Therefore, activation of GPR116 might represent a potential alternative to adiponectin replacement therapy.

In our in vivo and ex vivo data have shown that Gpr116 CKO mice exhibited more pronounced glucose intolerance, elevated systemic inflammation, impaired insulin response in peripheral tissues including liver and muscle, along with increased gene expression for the key genes to hepatic gluconeogenesis and lipogenesis. We hypothesized that abnormal expression and secretion of adipokines from adipocytes altered the endocrine functions and inflammation levels in adipose tissues which ultimately caused uncontrolled whole body glucose metabolism.

Taken together, in the current study, we investigated the role of GPR116 in adipose biology and systemic energy metabolism. GPR116 deficiency in adipocyte cell lines blocked the full differentiation of adipocytes, which is translated into dysfunctioning adipocytes in vivo, exemplified as smaller size, reduced adiponectin secretion and upregulated resistin. This study establishes GPR116 as a potential mediator linking adipose tissue dysfunction and systemic insulin resistance. GPR116 is an orphan receptor and the ligand for GPR116 is currently unknown. Fatty acids were found to be ligands for GPR116 and GPR120, the other two GPR proteins that regulate adipogenesis in 3T3-L1 cells [9–11]. In the case of GPR120, ω-3 fatty acid (ω-3 FA) was found to exert insulin-sensitizing effects when administered under the HFD conditions [11]. It is perceivable that the ligands for GPR116 would regulate insulin resistance once found. Clearly, identification of the natural ligand(s) for GPR116 is important in future works. Meanwhile, our findings shed new light on medications that act through rectification of adipose tissue dysfunction.

5. Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

6. Funding

This work was supported in part by grants from the National Basic Research Program of China (973 Program) (2011CB504004 and 2010CB945500), the Ministry of Science and Technology of China (2011BA115B02), the strategic program of stem cell (XDA01020303) and the National Science Foundation of China (30970637, 30810099, 90813033 and 30811120429).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.006.

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