ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 8, pp. 854-862. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 8, pp. 1050-1060.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-068, July 15, 2007.

Pentaspan Membrane Glycoprotein, Prominin-1, Is Involved in Glucose Metabolism and Cytoskeleton Alteration

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Received March 16, 2007
Revision received April 25, 2007

Abstract—This study constitutes the first report revealing the participation of prominin-1 in glucose metabolism and cytoskeleton alteration. Upon stimulation with high glucose, the expression of prominin-1 was up-regulated with concomitant down-regulation of its phosphorylation. Prominin-1 mutated at its phosphorylation site inflicted a global change in the expression of several genes associated with glucose metabolism in L6 myotube cells. Further, the over-expression of prominin-1 promoted glucose uptake in these cells. Prominin-1 undergoes sustained repression of its expression during confluence and differentiation of L6 myotube cells. The expression of prominin-1 in the MDCK cell modulated cell morphology and promoted cellular migration. These data imply that prominin-1 is involved in glucose metabolism and may regulate cellular glucose through its effect on cytoskeleton.

DOI: 10.1134/S000629790708007X

Key words: prominin-1, CD133/AC133, glucose, cytoskeleton

Prominin-1, a 115-120-kD membrane glycoprotein with five transmembrane loops, was first identified in mouse neuroepithelium [1] and human hematopoietic stem and progenitor cells [2, 3]. Prominin-1 was named after its prominent presence in membrane protrusion of the mouse cells. In human hematopoietic stem cells, it is known as CD133 (AC133), which shares about 60% identity with mouse prominin-1 [4]. Prominin is phylogenetically conserved across the animal kingdom such as in *Caenorhabditis elegans*, drosophila, zebra fish, and rodent. However, it is absent in the monoplast organism including *Saccharomyces cerevisiae* [5]. The scientific nomenclature has been proposed for this emergent protein family that comprises prominin-related sequences and their splice variants. This novel protein family is further divided into two classes, prominin-1 and prominin-2 [6].

Porminin-1 is characterized by its distinctive pentaspan membrane topology, with an N-terminal domain exposed to extracellular space, followed by two small cytoplasmic and two large extracellular loops, and a cytoplasmic C-terminal domain (Fig. 1). Seven potential N-glycosylation sites were identified, distributed on two extracellular loops, and are believed to serve as the recognition sites [7-9]. Irrespective of the cell type, prominin-1 displays a peculiar localization confined to microvilli and other protrusions or evaginations of plasma membrane [10, 11]. Prominin-1 resides in lipid raft, where it preferentially interacts with cholesterol [12]. Detailed information about the biological function of prominin-1 remains scarce; however, it is implicated in participation in construction and maintenance of plasma membrane protrusions [8]. The deletion of a single nucleotide in the human prominin-1 gene can cause inheritable retinal degeneration and impaired morphogenesis of photoreceptor disk [11]. Stem cells defined by their expression marker AC133 have been utilized to relieve symptoms of muscular dystrophy [13].

Prominin-1 was identified in our laboratory as a component of glucose homeostasis in a rat model [14, 15]

Abbreviations: CAMs) cell adhesion molecules; GSK3) glycogen synthase kinase-3; GST) glutathione S-transferase; MDCK) Madin–Darby canine kidney; PKC) protein kinase C; PPARγ) peroxisome proliferator activated receptor-γ.

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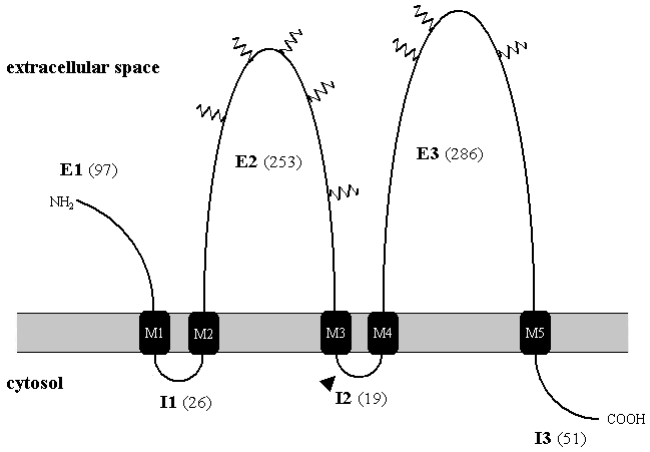

Fig. 1. Predicted membrane topology of prominin-1 (program TMHMM 2.0; CBS, Technical University of Denmark). Rat prominin-1 consists of an extracellular N-terminus (E1), five transmembrane segments (M1-M5) separating two small cytoplasmic loops (I1 and I2) and two large extracellular loops (E2 and E3), and a cytoplasmic C-terminus (I3). E2 and E3 contain all eight potential N-glycosylation sites. The numbers of amino acids residues in each domain are given in parentheses. The arrow indicates the putative phosphorylation site of protein kinase C. Domain I1 was used as bait protein to screen for binding partner proteins, and E2-GST fusion protein was used as the antigen to generate rabbit polyclonal antibodies.

that differed from the works of Weigmann [1] and Yin [2]. Several ESTs (expressed sequence tags) were cloned by differential display of rat skeletal muscle exposed to high versus normal concentrations of glucose. The full-length prominin-1 sequence was constructed following the correction of a reading frame error in the second extracellular loop [8]. Our previous research demonstrated that high blood glucose could induce the expression of prominin-1 in skeletal muscle of living rats, which in turn up-regulates GAPDH enzyme (3-phosphate glyceraldehyde dehydrogenase). Therefore, prominin-1 was classified as a glucose-responsive gene.

To investigate the molecular mechanism by which prominin-1 affects glucose metabolism, we used L6 myotubes, the insulin-responsive cells, as a model. In the present study, we have consolidated the involvement of prominin-1 in glucose regulation: (i) cellular mRNA expression and phosphorylation level of prominin-1 were changed considerably upon glucose exposure, (ii) the expression of prominin-1 in L6 myotubes induced uptake of glucose by these cells, and (iii) the expression of a prominin-1 mutant lacking its phosphorylation site modulated the transcription of many genes involved in glucose metabolism. In addition, a new feature of prominin-1 has been revealed by these results: prominin-1 expression in MDCK cells alters their morphology and migration potential.

MATERIALS AND METHODS

Plasmid construction. Rat prominin-1 cDNA was cloned as described in reference [15] (GenBank No.: NM021751). The bacterial expression plasmids pGEX-4T-1 containing the rat prominin-1 cDNA fragments from nucleotide 12 to 288, 525 to 1260, and 1488 to 2334, each fused in-frame to glutathione S-transferase (GST), were constructed by PCR amplification. The eukaryotic expression plasmid pcDNA3.1(+)-prominin-1 was used to produce a mutant (Thr459 to Ile459) lacking the phosphorylation site of protein kinase C (ΔPKC) by PCR mutagenesis using *Pyrobest* DNA polymerase (Takara, Japan).

Wild-type prominin-1 cDNA was introduced at *Hind*III and *Kpn*I restriction sites into pEGFP-N1 vector by means of high fidelity PCR. All amplified DNA fragments were sequenced to confirm the presence of mutation and the absence of any potential PCR error.

Polyclonal antibodies against recombinant prominin-1. GST-fusion protein containing the E2 fragment of prominin-1 (Arg175-Glu420) (Fig. 1) was expressed in BL21 *Escherichia coli* and purified using glutathione-Sepharose 4B beads as described by the manufacturer (Amersham Biosciences, USA). The fusion protein was purified in 10% polyacrylamide gel, homogenized, mixed (1 : 1) with complete Freund's adjuvant (Gibco, USA), and then injected subcutaneously into two New Zealand white rabbits. Immunization was repeated four to five times with 10-day intervals using Freund's incomplete adjuvant. The antibody (pAb E2) was purified from serum using ammonium sulfate precipitation. The affinity and specificity of this antibody was verified by enzyme-linked immunosorbent assay and Western blotting, respectively.

Cell culture and transfection. MDCK (Madin–Darby canine kidney) (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) and L6 cells (American Tissue Culture Collection) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C. Differentiation of L6 myoblasts to myotubes was induced in DMEM containing 2% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin (2.5 μg/ml) in a humidified incubator (37°C, 5% CO_2). The L6 myotubes were visualized and examined by staining with Giemsa. Cells were transfected with LipofectAMINE 2000 (Invitrogen, USA) following instructions from the manufacturer. The transformants were selected with G418 (500 μg/ml) for 3-4 weeks to establish G418-resistant cells. The results of L6 cells induction and transfection were observed under the fluorescent microscope and confirmed by Western blotting, respectively.

Cell migration assay. The migratory potential of MDCK cells stably expressing prominin-1 versus control was analyzed by migration assay, as described elsewhere

[17]. Briefly, the confluent cell monolayers were harvested using trypsin/EDTA and centrifuged at 800*g* for 5 min. The cell suspension was added to the upper chamber ($2 \cdot 10^5$ cells/well) of a pre-hydrated polycarbonate membrane filter (Costar Transwell, 6.5-mm diameter and 5-μm pore; Corning, USA). The lower and upper chambers contained the same medium. Cells were incubated in a humidified incubator with 5% CO_2 at 37°C for 24 h. The stationary cells on upside of the filter were scraped and washed. The cells that had migrated to the reverse side were stained with Giemsa and counted under the microscope. The experiments were repeated in triplicate.

Real-time PCR for assessment of mRNA levels. Total cellular RNA was isolated from cultured cells at the indicated times using the TRIZOL method (Invitrogen). The cDNA was synthesized from total RNA (2 μg) by reverse transcription using SuperScript First-Strand Synthesis System (Invitrogen). It was purified by means of QIAquick Nucleotide Removal Kit (Qiagen, USA). The mRNAs and the PCR products were assessed by real-time PCR with melting point analysis (ABI PRISM 7000; Perkin-Elmer Biosystems, USA). Real-time PCR samples were mixed with SYBR Green PCR Master Mix (Applied Biosystems, USA) and gene-specific primers. Thermal cycling conditions were as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Fluorescence intensity was recorded during each annealing step. Following primers were used to detect respective mRNAs: β-actin, 5′-TTCAACACCCCAGCCATGT-3′ (sense) and 5′-TGGTACGACCAGAGGCATACAG-3′ (anti-sense); and rat prominin-1, 5′-GGTTCGGCATAGGGAAAGC-3′ (sense) and 5′-GGCCAACTTGATAGCGATGATT-3′ (anti-sense).

Immunoprecipitation, Western blotting, and *in vitro* kinase assays. MDCK cells were lysed in 1% Nonidet P-40 buffer supplemented with protease inhibitors. The lysate were centrifuged at 12,000*g* for 10 min to remove insoluble materials, and protein concentrations were determined using BCA (Pierce, USA). For immunoprecipitation, aliquots of lysate were incubated for 2 h at 4°C with 1 : 300 dilution of pAb E2 or 1 : 100 dilution of monoclonal antibody HA (Sigma, USA) or p-Thr (Santa Cruz Biotechnology, USA). The resulting immunocomplexes were collected on protein G-Sepharose beads (Amersham Biosciences, USA), washed five times with 1% Nonidet P-40 lysis buffer, and subjected to 8% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane and incubated in blocking buffer for 2 h at room temperature. Rabbit polyclonal antibody E2 and p-Thr monoclonal antibody were prepared in blocking buffer at 1 : 3000 and 1 : 1000 dilutions, respectively.

After overnight incubation with the primary antibody at 4°C, the membrane was exposed to horseradish peroxidase-conjugated secondary antibody with gentle agitation for 2 h at room temperature. The bands were visualized by chemiluminescence using luminol reagent (Santa Cruz, USA). For PKC assays, pAb E2 was used to immunoprecipitate prominin-1 from MDCK cells. *In vitro* kinase reactions were carried out in 60 μl of PKC buffer for 5 min at 30°C (Promega, USA). The PKC was purified from rat brain and the PKC buffer consisted of 20 mM HEPES, 1 mM dithiothreitol (DTT), 1 mg/ml type III-S histone, 10 mM $MgCl_2$, 1.7 mM $CaCl_2$, and 0.2 mM [γ-^{32}P]ATP in the presence and absence of 600 μl/ml phosphatidylserine. Reactions were terminated by SDS sample buffer, and proteins were subjected to electrophoresis.

Measurement of glucose uptake. The L6 myotubes transfected with pcDNA3.1-prominin-1 or pcDNA3.1 (control) were washed twice with PBS and incubated for 3 h in 1% BSA/PBS under humidified atmosphere (37°C, 5% CO_2). Starved cells were exposed to 0.5 or 100 mM (unspecific absorption control) 2-deoxy-D-[2,6-^{3}H]glucose (1 μCi/ml; Amersham Biosciences) for 15 min. The samples were washed 4× with ice-cold PBS. Cells lysates were prepared in 1% Triton X-100 and mixed with 5 ml scintillation cocktail (0.1 mg/ml POPOP, 5 mg/ml PPO, 66% methylbenzene, and 34% Triton X-100) for radioactivity measurement.

Confocal microscopy. Cells were incubated sequentially with the primary antibody and TRITC-conjugated secondary antibody in a humidified chamber (30 min, 37°C). For F-actin staining, cells were treated with phalloidin-TRITC (50 μg/ml; Sigma, USA) for 40 min at room temperature. All incubation and washes were performed directly on the cover slips. 1,4-Diazabicyclo[2,2,2]octane was used to alleviate fluorescence quenching before sealing the cover slips with glycerol. Cells were examined by confocal laser-scanning microscope (Olympus FV500). The images were compiled using the Adobe Photoshop v. 6.0 software.

RESULTS

Up-regulation of prominin-1 mRNA is more robust in response to high glucose than insulin. The mRNA expression of prominin-1 in L6 myotubes was evaluated to further corroborate our previous experiments [15]. Based on the assumption that prominin-1 might be involved in insulin-regulated glucose metabolism, we tested the comparative effects of insulin and high glucose on L6 cells. The preincubation of L6 myotubes in 1% BSA/PBS for 2 h minimized the undesired influence of fetal bovine serum and existing glucose in the medium. The results of real-time PCR were normalized to β-actin transcript and depicted in Fig. 2. The transcription of prominin-1 mRNA was greater and significantly increased in response to high glucose than insulin stimulation, particularly at 60 min. During this period, glucose-induced prominin-1 transcript increased 5- or 40-fold, in relation to insulin or PBS, respectively. To identify the mechanism, L6 cells

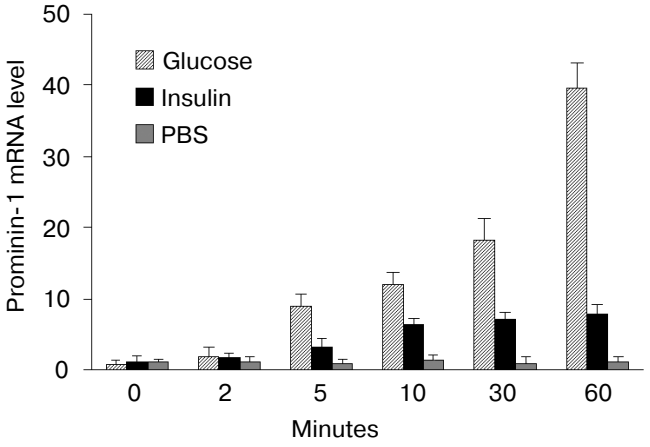

Fig. 2. Expression of prominin-1 mRNA upon stimulation with high glucose or insulin. Differentiated L6 myotubes were incubated in 1% BSA/PBS for 2 h and then treated with glucose (80 mM), insulin (10 nM), or PBS for the indicated duration. Reverse transcription and real-time PCR were performed using β-actin as a control. The results are depicted as the means ± SD of three independent experiments.

were pretreated with a transcriptional inhibitor, actinomycin D, for 30 min *prior to* glucose. Surprisingly, actinomycin D completely abolished the glucose-triggered increment in prominin-1 mRNA (data not shown) indicating its transcriptional regulation by glucose. The upregulation of prominin-1 by high glucose is consistent with our previous data on differential display in SD rats. The fact that prominin-1 was also induced by insulin provides a basis for further investigation on the function of prominin-1 in regulation of glucose metabolism.

Differential phosphorylation profiles of prominin-1 in response to high glucose and insulin and multiple phosphorylations by PKC. The phosphorylation of signaling molecules by protein kinase C (PKC) and their dephosphorylation are some of the most universal phenomena in signal transduction. Therefore, prominin-1 sequence was analyzed for PKC phosphorylation sites. PROSITE database (website http://www.expasy.ch/prosite/) was employed to analyze prominin-1 and one potential residue (Thr459) was identified on the cytoplasmic I2 segment. *In vitro* PKC phosphorylation was conducted to validate the software analysis. Prominin-1 mutant (ΔPKC, Thr459Iso) devoid of phosphorylation site was constructed by site-directed mutagenesis, and expressed in MDCK cells. Equal amounts of wild-type prominin-1 and ΔPKC as estimated by Coomassie blue-stained SDS-PAGE were immunoprecipitated by pAb E2 (data not shown). The titer of pAb E2 was 1 : 20,000 as determined by ELISA. Type III-S histone served as a positive control. Prominin-1 was evidently phosphorylated by PKC (120-kD band, Fig. 3a). Surprisingly, a weak autoradiography signal was observed for ΔPKC that implicated the presence of other phosphorylation sites on prominin-1.

Prominin-1 was identified in the skeletal muscle of high glucose-treated SD rats using the mRNA differential display method [15]. Hence, we were prompted to determine if the phosphorylation status of prominin-1 was also affected by excessive glucose. We utilized the L6 myotube that represent an excellent model for the intact muscle and its glucose metabolism. Serum-starved and glucose-free L6 myotubes cultures were incubated with high glucose for the indicated times (Fig. 3b). Immunoprecipitated prominin-1 was detected and quantified for its phosphorylation using the antibody against p-Thr. As depicted in Fig. 3, phosphorylation of threonine first increased slightly and then declined sharply during glucose stimulation. Skeletal muscle is one of the three major target tissues of insulin action on glucose metabolism, which raises the possibility of prominin-1 phosphorylation in L6 myotubes in response to insulin. However, we failed to detect the phosphorylation of threonine in insulin-treated L6 cells.

ΔPKC modulates expression of genes associated with glucose metabolism. The phosphorylation pattern of prominin-1 is affected by high glucose; hence, we anticipated the regulatory effect of phosphorylation mutant of prominin-1 (ΔPKC) on the cellular metabolism of glucose.

Real-time PCR was employed to investigate whether ΔPKC would alter the expression of qualified genes in L6 myotubes. Wild-type prominin-1 and β-actin were used as standard and normalization controls, respectively. In total, 39 genes involved in insulin action, glucose and lipid metabolism, lipid raft, and cytoskeleton motility were evaluated for the influence of ΔPKC. The expression level of 7 out of 39 genes was changed readily (table). Most of these gene products are key participants in glucose metabolism. These results further consolidated the relationship between prominin-1 and glucose metabolism.

Gene modulation in response to expression of wild-type prominin-1 and its phosphorylation mutant (ΔPKC) in L6 myotubes

Gene	ΔPKC/prominin-1 ratio
Glycogen synthase kinase 3	0.29
Hexokinase	3.3
Aldolase B	0.22
Aldose reductase 1	0.04
PKCζ (protein kinase C ζ)	0.11
PI3K p110	2.5
Akt/PKB (protein kinase B)	0.23

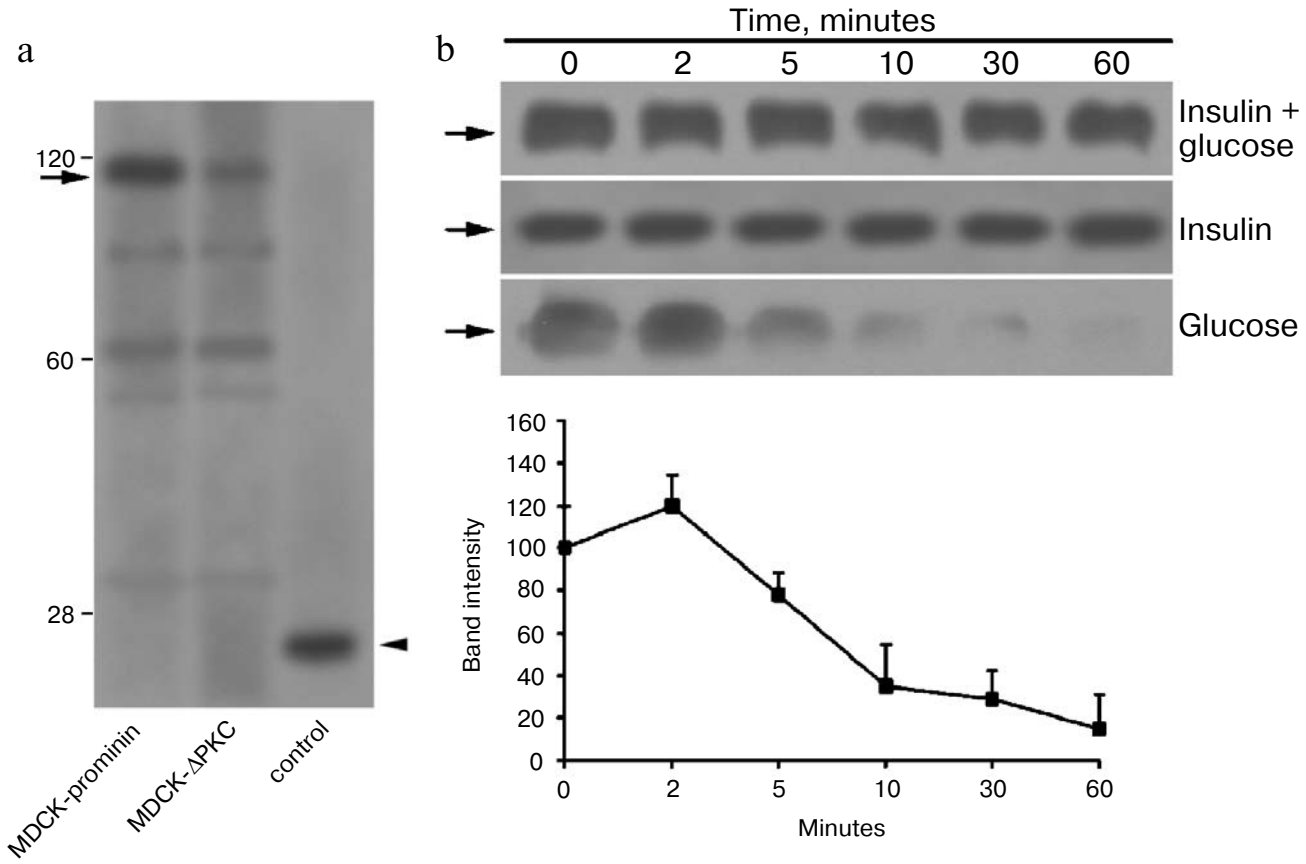

Fig. 3. Phosphorylation of prominin-1. The polyclonal antibody E2 (pAb E2) was used to precipitate prominin-1 from cell lysate for subsequent phosphorylation. a) *In vitro* phosphorylation analysis. The wild-type prominin-1 (MDCK-prominin) and its mutant (Thr459Ile) lacking the putative phosphorylation site of protein kinase C (MDCK-ΔPKC) were precipitated from recombinant MDCK cells stably expressing these proteins, and then subjected to *in vitro* phosphorylation as described under "Materials and Methods". Proteins recovered from the phosphorylation reactions were separated on an 8% SDS-polyacrylamide gel and visualized by autoradiography. b) Phosphorylation profile of prominin-1 in L6 myotubes upon stimulation with glucose, insulin, and glucose along with insulin. Differentiated L6 myotubes were incubated in 1% BSA/PBS for 2 h *prior to* time-dependent treatment with glucose (80 mM) or insulin (10 nM). Equal amounts of prominin-1 obtained by pAb E2 were stained with p-Thr monoclonal antibody. The graph illustrates the quantification of three independent glucose stimulation assays. The results are shown as means ± SD. Arrow, prominin-1; arrowhead, type III-S histone.

Expression of prominin-1 in L6 myotubes induces glucose uptake. Expression of prominin-1 as well as its phosphorylation profile were altered by incubation with high concentration of glucose, which indicates the interrelationship between both of these phenomena. Based on the assumption that a positive feedback mechanism assists an organism to import and store abundant blood glucose, its uptake was measured in L6 myotubes. The L6 myoblasts were transfected either with pcDNA3.1-prominin-1 or vector, cultured for four weeks in presence of G418 to select for the stable clones, and subsequently induced to L6 myotubes. 2-Deoxy-D-[2,6-^{3}H]glucose (1 μCi/ml) was used to determine the glucose uptake by differentiated myotubes. The L6 myotubes expressing prominin-1 demonstrated 1.8-fold elevated protein expression, as determined by the Western blot analysis. As anticipated, these cells also exhibited (1.6 ± 0.13)-fold higher import of glucose than control ($p < 0.05$) (Fig. 4). These data reveal that higher expression and reduced phosphorylation of prominin-1 in presence of glucose is indicative of certain tissue response to excessive sugar in its microenvironment.

Down-regulation of prominin-1 during differentiation of L6 cells. AC133, a human homolog of rat prominin-1, is reported to be down-regulated during the differentiation of human epithelial cell line Caco-2 [10]. To determine if the rat prominin-1 would also reveal a similar pattern during differentiation of rat L6 cells, the mRNA transcription and protein expression of prominin-1 were monitored using real-time PCR and Western blot, respectively. The L6 myoblasts upon their culture in 2% fetal bovine serum for 6-8 days differentiate into myotubes according to the morphological marker of multi-nuclei cells and transcriptional marker of myogenin [16] that would subsequently undergo senescence. The expression of prominin-1 was also examined *prior to* confluence to

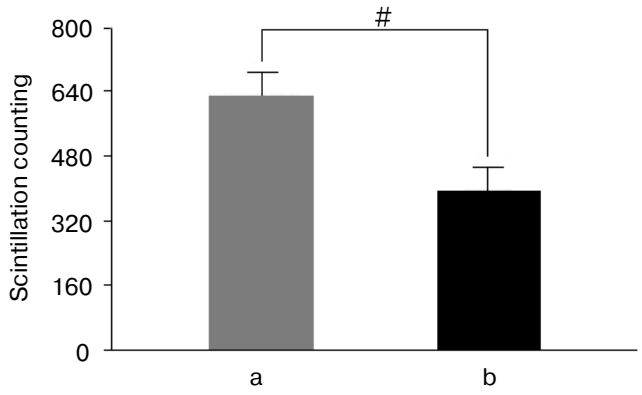

Fig. 4. Effect of prominin-1 expression on glucose uptake by L6 myotubes. L6 myotubes stably transfected with pcDNA3.1-prominin-1 (a) demonstrated (1.6 ± 0.13)-fold higher glucose import than control cells harboring pcDNA3.1 vector (b). The results are shown as means ± SD of three experiments; $^{\#}p < 0.05$.

determine the effect of cell-to-cell contact. β-Actin served as loading control. As shown in Fig. 5d, protein expression of prominin-1 was highest just *prior to* confluence and decreased gradually until the 6th day. Interestingly, prominin-1 level gained after day 6 of induction; however it remained below day 0. The determination of prominin-1 expression after day 12 could not be performed due to death of most L6 myotubes. The pAb E2 made in our laboratory was used in all above-mentioned experiments; therefore, the efficacy of the antibody on a Western blot has also been included in Fig. 5a.

Prominin-1 expression in MDCK cells alters their morphology and migration. Being a eukaryotic cell devoid of endogenous prominin-1, the MDCK cell culture offers an excellent model to study the biological function of prominin-1 [7]. To monitor the modulatory effect of prominin-1 on the cytoskeleton, we generated a MDCK cell line stably expressing prominin-1 (MDCK-prominin-1). Significant changes in the morphology of MDCK-prominin-1 were observed compared to control MDCK cells harboring the empty vector (Figs. 6a and 6b).

Before confluence, MDCK cells aggregated in piles with an elliptical outline. In contrast, MDCK-prominin-1 cells were dispersed in cultures with a large and slim border, especially with one or more long protrusions. The edges of confluent MDCK cells, however, were more indistinct than MDCK-prominin-1 exhibiting a sharp outline. To further investigate if these changes in cell shape were due to prominin-1-induced actin remodeling, TRITC-conjugated phalloidin was used to visualize F-actin (Figs. 6c and 6d). Identical observations were made in stained and unstained cells under the light microscopy with no noticeable cytoskeleton alterations.

Prominin-1 exhibits a few properties of the cell adhesion molecules (CAMs): e.g. the selective localization on protrusions of plasma membrane [1], participation in polarization and migration of $CD34^{+}$ hematopoietic cells [18], and its down-regulation during cell conflu-

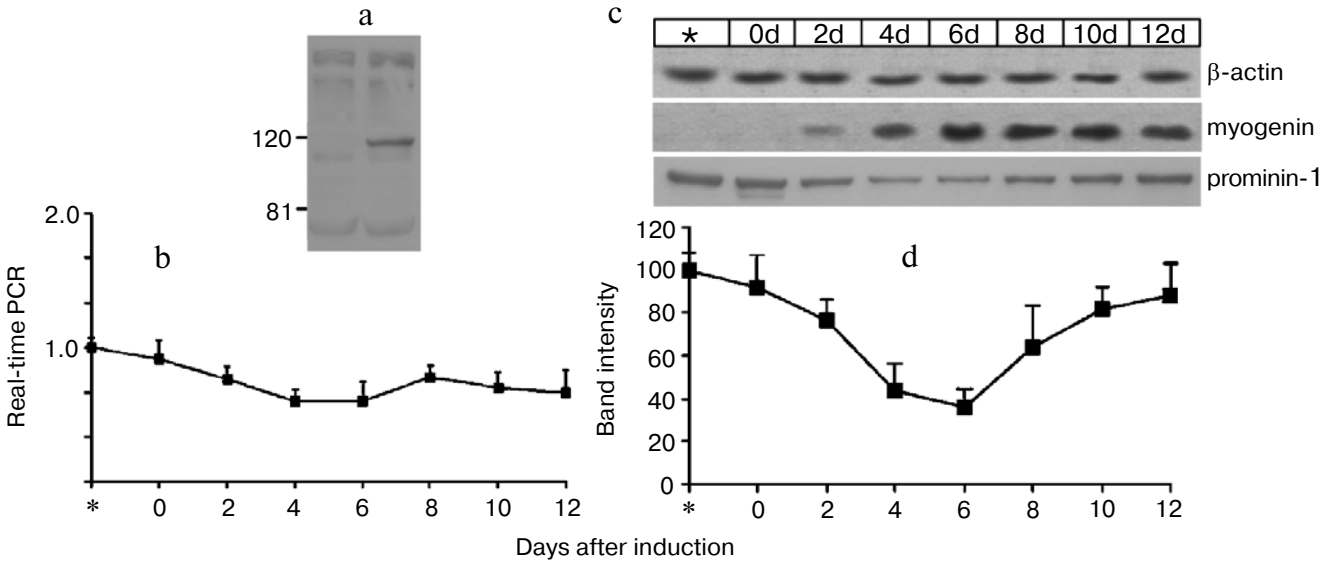

Fig. 5. Temporal modulation of prominin-1 expression during the differentiation of L6 cells. a) Prominin-1 in MDCK cells was detected by Western blot using pAb E2. Left lane, MDCK cells; right lane, MDCK cells expressing prominin-1. b) Real-time PCR of prominin-1 mRNA during L6 differentiation. c) Equal amounts of BCA-estimated total proteins from L6 cells were subjected to SDS-PAGE and Western blot analysis using pAb E2. Myogenin and β-actin served as L6 cell differentiation marker and loading control, respectively. The picture represents three independent experiments. d) Semi-quantitative estimation of prominin-1 from panel (c) using BIO-RAD Quantity One software. The results are depicted as means ± SD. Asterisk, L6 cells *prior to* confluence; 0d, the day *prior to* induction (i.e. 80% confluence).

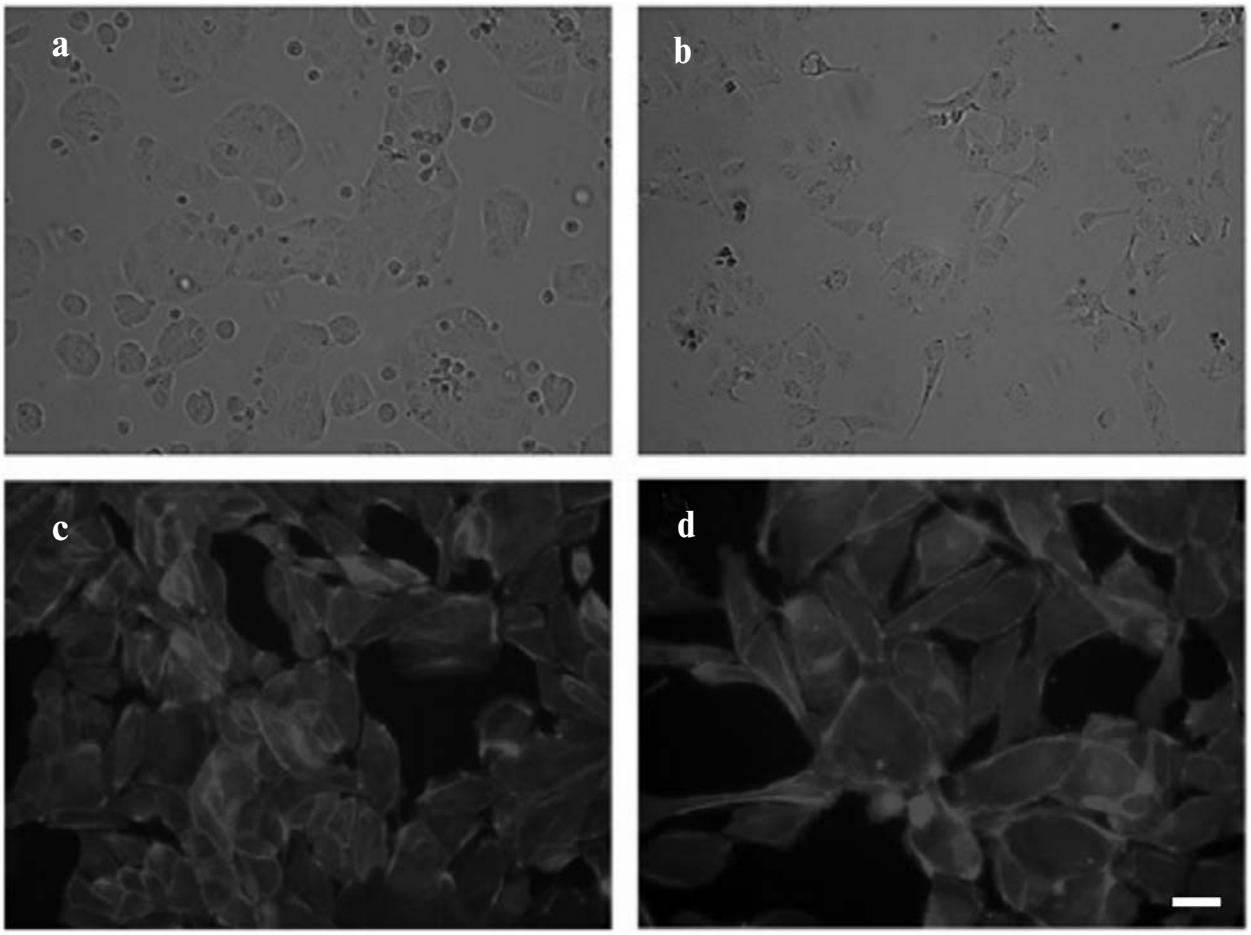

Fig. 6. Morphological changes in MDCK due to heterologous expression of prominin-1. a, c) MDCK cells; b, d) MDCK cells expressing prominin-1. Cells were cultured in 96-well plate until subconfluence and photographed. a, b) Phase-contrast microscopy; c, d) fluorescence microscopy after phalloidin-TRITC staining. The panels are adjusted to the same magnification. Bar, 10 μm.

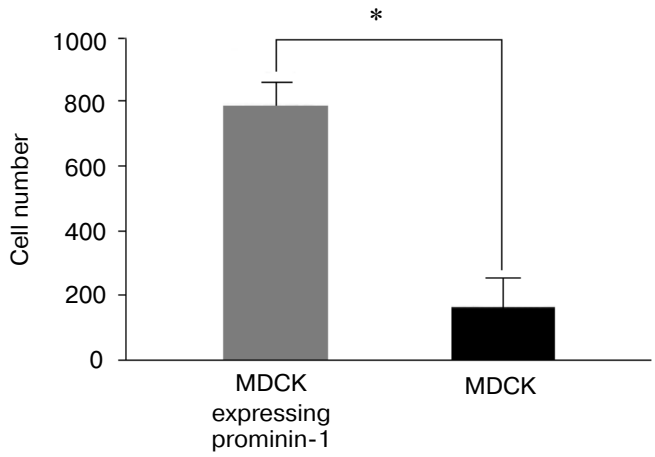

Fig. 7. Effect of prominin-1 expression on migratory capacity of MDCK cells. The migration potential of MDCK cells was measured by Giemsa staining. Cells expressing prominin-1 (MDCK-prominin-1) exhibited 4-fold higher movement compared to control (MDCK). The results are means ± SD of three separate experiments; * $p < 0.01$.

ence. To elucidate the role of prominin-1 in cell motility, we performed the cell migration assay on a polycarbonate membrane filter (5-μm pore) fitted in a Transwell. MDCK and MDCK-prominin-1 cells were counted under a microscope after overnight incubation (Fig. 7). In accordance with our presumption, the expression of prominin-1 facilitated the cell migration capacity of MDCK-prominin-1 that was about 4-fold higher than MDCK control cells.

DISCUSSION

In the present study, we have demonstrated that prominin-1 exhibits two distinct physiological features: (i) it is a glucose-responsive gene, and (ii) it participates in cytoskeleton alteration. The former aspect is revealed by the fact that the expression and phosphorylation pattern of prominin-1 were affected in the presence of high glucose, and the over-expression of prominin-1 promoted glucose import. The second characteristic is supported by

the data that the expression of prominin-1 altered the shape and migration capacity of cells.

Knowledge about the function and physiological relevance of prominin-1 is limited, but has been growing rapidly in recent years. In the early stage, this protein drew considerable attention of scientists because of its unique sequence, membrane topology, subcellular localization, and its association with lipid raft. This five-membrane-span glycoprotein demonstrates almost constitutive and ubiquitous expression, and is believed have important physiological functions. Unfortunately, the current knowledge of prominin-1 is limited to its localization and expression profiles in various tissues. Prominin-1 is strictly confined to the apical surface of various types of cells including epithelial, hematocyte, photoreceptor, and epididymal duct [9, 19]. Although, prominin-1 is highly conserved in the animal kingdom, it has numerous alternative splice variants and distinct glycosylation patterns in different tissues along with development-specific expression [5, 20]. CD133/AC133, the human homolog of prominin-1, can be used as a marker to identify hematopoietic stem and progenitor cells. Recently, $CD133^+$ cells were also detected in differentiated solid tissues and kidney cancer indicating the potential of CD133 epitope as a diagnostic marker to investigate solid cancers [21-23]. Intriguingly, Marzesco and colleagues have reported that CD133 can be released into the extracellular fluids along with membrane particles [24]. All these findings do not, however, assign a conclusive function to prominin-1 protein.

Using the differential display method, we identified prominin-1 in skeletal muscle of a rat model designed to study blood glucose homeostasis. Prominin-1 was originally named Fudenine after a mistaken adenine deletion in the cDNA screened from a library. This deletion generated a premature stop codon resulting in a truncated protein with only 591 residues compared to rat prominin (857 amino acids) [15]. Corbeil et al. discovered this protein not to be a splice variant of prominin-1, but a cloning mistake instead [8].

The expression of prominin-1 in skeletal muscles increased upon a 30 min transfusion of a living rat with concentrated glucose solution. Here, the L6 myotube, which is an excellent cell model to investigate glucose metabolism and insulin action, was employed to study prominin-1 [25]. The same treatments were repeated in L6 myotubes to validate the up-regulation of prominin-1 upon glucose stimulation (Fig. 2). The minor impact of insulin indicates that it has a rather weak effect on prominin-1 expression. A high osmotic pressure environment stimulated by sorbitol was used as a control in an independent experiment to eliminate the prospect of misleading results that may have been caused by glucose-related hyper-osmotic condition (data not shown). Thus, glucose itself, not the sugar-induced hyperosmotic phenomenon, triggers the up-regulation of prominin-1 in animal as well as in the cell models.

To further investigate the biological function of prominin-1 in glucose metabolism, experiments were designed to detect bidirectional glucose transport. The L6 myotubes with over-expressed prominin-1 exhibited (1.6 ± 0.13)-fold higher glucose uptake than cells transfected with vector. In addition, the transcripts of many genes related to glucose metabolism (GSK3, hexokinase, aldolase B, aldose reductase 1, PKCζ, and Akt/PKB) were substantially affected upon the expression of prominin-1 mutated at its phosphorylation site. These results provide strong evidence that prominin-1 is involved in cellular glucose metabolism. The phosphorylation level of prominin-1 was increased slightly within 2 min of glucose stimulation, and subsequently declined suggesting that phosphorylation of prominin-1 may regulate glucose uptake. The fact that phosphorylation was first increased modestly and it could be reversed by insulin demands more investigation to elucidate this phenomenon.

PPARγ (peroxisome proliferator activated receptor-γ) has been reported to regulate the storage of excess energy substrate for future usage [26]. Unexpectedly, PPARγ showed no correlation with prominin-1 in our luciferase reporter assay (data not shown). However, the down-regulation of GSK3 (glycogen synthase kinase-3) upon the expression of phosphorylation site-deficient prominin-1 would still support the assumption that prominin-1 participates in glucose uptake and energy deposit. The repression of GSK3 may serve as a mechanism that compensates for the absence of phosphorylation on prominin-1, and produces more glycogen to deposit excess energy. Therefore, prominin-1 can be assumed as a glucose-responsive gene for its regulatory effect on glucose transport.

Previous studies have demonstrated that expression of prominin-1/CD133 was decreased concomitant with the confluence or differentiation of cells [5, 10, 22]. In this study, a similar phenomenon was observed during the confluence and induction from L6 myoblasts to myotubes. No significant changes occurred in mRNA level of prominin-1 implicating its translational or post-translational down-regulation.

Besides the potential effect on membrane protrusion, prominin-1 may also influence cell polarity, migration, and interaction with the neighboring cells and/or extracellular matrix [5, 18]. So far, prominin-1 has been identified only in multi-cellular organisms, with no detectable expression in monoplast organisms including yeast. Our data indicate that expression of prominin-1 in MDCK leads to obvious changes in cell morphology and their higher migration capacity. Prominin-1, therefore, exhibits the characteristics of cell adhesion molecules, e.g. cell transformation, chemotaxis, migration, and recognition. Recent evidences suggest that cytoskeleton movement and actin remodeling play an important role in glucose metabolism and insulin action [27-29]. Collectively, it can be proposed that prominin-1 would

promote glucose uptake through its modulatory effect on cytoskeleton. This hypothesis awaits additional experimentation, however.

In conclusion, this study provides the first evidence of the potential biological function of prominin-1. This protein participates in glucose metabolism and cytoskeleton alteration, presumably through a mechanism that regulates glucose in a microenvironment with excessive sugar. Together, these data endorse prominin-1 as a potential target for regulation of blood glucose.

We thank Dr. Denis Corbeil (Max-Planck Institute of Molecular Cell Biology and Genetics, Germany) for his critical suggestions. We are also grateful to Dr. Chengyu Jiang (National Laboratory of Medical Molecular Biology, PUMC&CAMS, China), and Wenjun Shen (Department of Medicine, Stanford University, USA) for their valuable comments and discussion.

This work was supported by the grants "973" Project of China (No. 2004CB518602 and No. 2006CB503909) and National Natural Science Foundation of China (No. 30471930).

REFERENCES

1. Weigmann, A., Corbeil, D., Hellwig, A., and Huttner, W. B. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 12425-12430.
2. Yin, A. H., Miraglia, S., Zanjani, E. D., Almeida-Porada, G., Ogawa, M., Leary, A. G., Olweus, J., Kearney, J., and Buck, D. W. (1997) *Blood*, **90**, 5002-5012.
3. Miraglia, S., Godfrey, W., Yin, A. H., Atkins, K., Warnke, R., Holden, J. T., Bray, R. A., Waller, E. K., and Buck, D. W. (1997) *Blood*, **90**, 5013-5021.
4. Fargeas, C. A., Corbeil, D., and Huttner, W. B. (2003) *Stem Cells*, **21**, 506-508.
5. Shmelkov, S. V., Clair, St. R., Lyden, D., and Rafii, S. (2005) *Int. J. Biochem. Cell Biol.*, **37**, 715-719.
6. Fargeas, C. A., Florek, M., Huttner, W. B., and Corbeil, D. (2003) *J. Biol. Chem.*, **278**, 8586-8596.
7. Corbeil, D., Roper, K., Hannah, M. J., Hellwig, A., and Huttner, W. B. (1999) *J. Cell. Sci.*, **112**, 1023-1033.
8. Corbeil, D., Fargeas, C. A., and Huttner, W. B. (2001) *Biochem. Biophys. Res. Commun.*, **285**, 939-944.
9. Fargeas, C. A., Joester, A., Missol-Kolka, E., Hellwig, A., Huttner, W. B., and Corbeil, D. (2004) *J. Cell Sci.*, **117**, 4301-4311.
10. Corbeil, D., Roper, K., Hellwig, A., Tavian, M., Miraglia, S., Watt, S. M., Simmons, P. J., Peault, B., Buck, D. W., and Huttner, W. B. (2000) *J. Biol. Chem.*, **275**, 5512-5520.
11. Maw, M. A., Corbeil, D., Koch, J., Hellwig, A., Wilson-Wheeler, J. C., Bridges, R. J., Kumaramanickavel, G., John, S., Nancarrow, D., and Roper, K. (2000) *Hum. Mol. Genet.*, **9**, 27-34.
12. Roper, K., Corbeil, D., and Huttner, W. B. (2000) *Nat. Cell Biol.*, **2**, 582-592.
13. Torrente, Y., Belicchi, M., Sampaolesi, M., Pisati, F., Meregalli, M., D'Antona, G., Tonlorenzi, R., Porretti, L., Gavina, M., and Mamchaoui, K. (2004) *J. Clin. Invest.*, **114**, 182-195.
14. Zhu, G., Zuo, J., and Fang, F. (2000) *Chin. Med. J.*, **113**, 894-898.
15. Zhu, G., Chang, Y., Zuo, J., Dong, X., Zhang, M., Hu, G., and Fang, F. (2001) *Biochem. Biophys. Res. Commun.*, **281**, 951-956.
16. Nervi, C., Benedetti, L., Minasi, A., Molinaro, M., and Adamo, S. (1995) *Cell Growth Differ.*, **6**, 81-89.
17. Yue, T. L., McKenna, P. J., Ohlstein, E. H., Farach-Carson, M. C., Butler, W. T., Johanson, K., McDevitt, P., Feuerstein, G. Z., and Stadel, J. M. (1994) *Exp. Cell Res.*, **214**, 459-464.
18. Giebel, B., Corbeil, D., Beckmann, J., Hohn, J., Freund, D., Giesen, K., Fischer, J., Kogler, G., and Wernet, P. (2004) *Blood*, **104**, 2332-2338.
19. Shmelkov, S. V., Jun, L., Clair, St. R., McGarrigle, D., Derderian, C. A., Usenko, J. K., Costa, C., Zhang, F., Guo, X., and Rafii, S. (2004) *Blood*, **103**, 2055-2061.
20. Corbeil, D., Roper, K., Fargeas, C. A., Joester, A., and Huttner, W. B. (2001) *Traffic*, **2**, 82-91.
21. Richardson, G. D., Robson, C. N., Lang, S. H., Neal, D. E., Maitland, N. J., and Collins, A. T. (2004) *J. Cell Sci.*, **117**, 3539-3545.
22. Florek, M., Haase, M., Marzesco, A. M., Freund, D., Ehninger, G., Huttner, W. B., and Corbeil, D. (2005) *Cell Tissue Res.*, **319**, 15-26.
23. Kania, G., Corbeil, D., Fughs, J., Tarasov, K. V., Blyszczuk, P., Huttner, W. B., Boheler, K. R., and Wobus, A. M. (2005) *Stem Cells*, **23**, 791-804.
24. Marzesco, A. M., Janich, P., Wilsch-Brauninger, M., Dubreuil, V., Langenfeld, K., Corbeil, D., and Huttner, W. B. (2005) *J. Cell Sci.*, **118**, 2849-2858.
25. Gerrard, D. E., and Judge, M. D. (1993) *J. Anim. Sci.*, **71**, 1464-1470.
26. Fabris, R., Nisoli, E., Lombardi, A. M., Tonello, C., Serra, R., Granzotto, M., Cusin, I., Jeanrenaud, F. R., Federspil, G., Carruba, M. O., and Vettor, R. (2001) *Diabetes*, **50**, 601-608.
27. Chang, L., Chiang, S. H., and Saltiel, A. R. (2004) *Mol. Med.*, **10**, 65-71.
28. Eyster, C. A., Duggins, Q. S., and Olson, A. L. (2005) *J. Biol. Chem.*, **280**, 17978-17985.
29. Strawbridge, A. B., and Elmendorf, J. S. (2005) *Diabetes*, **54**, 1689-1705.