FEBS Letters

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Pyruvate kinase M2 represses thermogenic gene expression in brown adipocytes

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(Received 18 September 2019, revised 16 November 2019, accepted 2 December 2019, available online 20 December 2019)

doi:10.1002/1873-3468.13716

Edited by László Nagy

Utilizing the thermogenic capacity of brown adipose tissue is a potential anti-obesity strategy; therefore, the mechanisms controlling expression of thermogenesis-related genes are of interest. Pyruvate kinase (PK) catalyzes the last step of glycolysis and exists as four isoenzymes: PK, liver, PK, red blood cell, PK, muscle (PKM1 and PKM2). PKM2 has both glycolytic and nuclear functions. Here, we report that PKM2 is enriched in brown adipose compared with white adipose tissue. Specific knockdown of PKM2 in mature brown adipocytes demonstrates that silencing of PKM2 does not lead to a decrease in PK activity, but causes a robust upregulation of thermogenic uncoupling protein 1 (*Ucp1*) and fibroblast growth factor 21 (*Fgf21*) gene expression. This increase is not mediated by any of the known mechanisms for PKM2-regulated gene expression, thus implying the existence of a novel mechanism for PKM2-dependent effects on gene expression.

Keywords: brown adipose tissue; Fgf21; glycolysis; metabolism; obesity; pyruvate kinase; thermogenesis; Ucp1

Brown adipose tissue (BAT) is specialized in nonshivering thermogenesis [1,2]. The protein responsible for this process is uncoupling protein 1 (UCP1) that is located in the inner mitochondrial membrane where it uncouples the electron transport chain and thus oxygen consumption from ATP production. BAT is important for rodents to defend their body temperature in response to prolonged cold exposure and has been demonstrated to counteract obesity [2,3], and the presence and activity of BAT in humans is now well established [1]. In addition to *Ucp1*, thermogenesis-related genes that are enriched in brown and brown-like adipocytes include fibroblast growth factor 21 (*Fgf21*), cell death-inducing DFFA-like effector a (*Cidea*), type 2 iodothyronine deiodinase (*Dio2*), and peroxisome proliferator-activated receptor γ coactivator 1α (*Ppargc1a*) [4–8]. Regulation of these 'thermogenic' genes is important to understand, as recruitment of thermogenic capacity of BAT is considered an anti-obesity strategy [9].

We have previously reported an extensive regulation of genes encoding enzymes involved in glucose metabolism, including glycolysis, in BAT from cold-exposed

Abbreviations
BAT, brown adipose tissue; CCND1, cyclin D1; CIDEA, cell death-inducing DFFA-like effector a; CTNNB1, β-catenin; DIO2, type 2 iodothyronine deiodinase; FGF21, fibroblast growth factor 21; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporter; LDH, lactate dehydrogenase; MEK5, mitogen-activated protein kinase kinase 5; MYC, v-myc avian myelocytomatosis viral oncogene homolog; PK, pyruvate kinase; PKL, PK, liver; PKM, PK, muscle; PKR, PK, red blood cell; PPARGC1A, peroxisome proliferator-activated receptor γ coactivator-1α; RT–qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; siRNA, small interfering RNA; TBP, TATA-binding protein; TFIIB, transcription factor II B; UCP1, uncoupling protein 1.

mice [10,11]. In the present study, we have specifically investigated pyruvate kinase (PK), the enzyme catalyzing the final step of glycolysis, and the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. Four mammalian PK isoenzymes exist as follows: PK, liver (PKL) (liver isoform), PK, red blood cell (PKR; red blood cell isoform), PKM1 and PKM2 (muscle isoforms). PKL and PKR are expressed in liver and erythrocytes, respectively, and are encoded by the same gene (*Pklr*), but are expressed under the control of different tissue-specific promoters [12]. PKM1 and PKM2 are generated by alternative splicing of transcripts from the *Pkm* gene and differ by only one exon encoding 56 amino acids [13]. PKM1 is expressed in tissues where large amounts of energy have to be provided rapidly, for example, skeletal muscle and brain. PKM2 was earlier thought only to be expressed during embryogenesis, but has later been established to also be expressed in many cancers and some differentiated tissues, that is, lung, pancreatic islets, and adipose tissue [14,15]. PKM2 is considered important for aerobic glycolysis, that is, conversion of glucose to lactate under normoxic conditions, in cancer cells, a phenomenon known as the Warburg effect [16]. Even though PKM2 is a cytosolic enzyme involved in glycolysis, it also contains an inducible nuclear localization signal [17]. In the nucleus, PKM2 can act as a cofactor for several transcription factors [18] and as a dual-specificity protein kinase [19].

KM1 and PKM2 possess both overlapping and nonoverlapping functions. Expression of both PKM isoforms is increased by thermogenic activation in BAT [10,11]. Here, we have investigated the significance of the two isoforms by specifically depleting PKM1 or PKM2 in mature brown adipocytes. We found that knockdown of PKM2, but not PKM1, augmented the expression of selected thermogenic genes.

Materials and methods

Mouse tissues

Tissues were obtained from five 3-month-old female C57BL/6 mice. Dissected tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Culture of cell lines

The WT-1 cell line was kindly provided by C. Ronald Kahn [20]. It was established by immortalization of brown preadipocytes from newborn mice with simian virus 40 large T antigen. WT-1 cells were propagated in Dulbecco's modified Eagle's medium (#52100; Life Technologies, Naerum, Denmark) supplemented with 10% FBS (#10270-106; Life Technologies), 62.5 $\mu g \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin (#15140-122; Life Technologies). Two days postconfluent, WT-1 cells (designated day 0) were induced to differentiate in propagation medium supplemented with 1 μM dexamethasone (#D1756; Sigma-Aldrich, Soeborg, Denmark), 0.5 mM 3-isobutyl-1-methylxanthine (#I5879; Sigma-Aldrich), 5 $\mu g \cdot mL^{-1}$ insulin (#11376497001; Roche, Hvidovre, Denmark), and 1 μM rosiglitazone (#71740; Cayman Chemical, Ann Arbor, MI, USA). At day 2, cells were refreshed with medium containing 5 $\mu g \cdot mL^{-1}$ insulin and 1 μM rosiglitazone. From day 4, the cells were cultured in WT-1 propagation medium without the supplements. Cell cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2.

Isolation and culture of primary adipocytes

Primary brown adipocytes from interscapular, cervical, and axillary BAT from 3- to 4-week-old NMRI mice of either sex were isolated as described [21]. The cell pellet was resuspended in culture medium and plated in 6-well plates. Cultures were incubated in a humidified atmosphere of 8% CO_2 at 37 °C. The cell culture medium was changed at days 1, 3, 5, and 7 after plating.

Replating and reverse small interfering RNA transfection of mature adipocytes

Mature adipocytes (day 6 for WT-1 cells and day 8 for primary brown cultures) were reverse-transfected with small interfering RNAs (siRNAs) as described [21]. The final concentrations of transfection reagent and siRNA were 5 $\mu L \cdot mL^{-1}$ and 50 nM, respectively, for WT-1 cells replated into 96-well plates, and 9 $\mu L \cdot mL^{-1}$ and 90 nM, respectively, for primary brown adipocytes replated into 48-well plates. Mature adipocytes were harvested 4 days after transfection. The MISSION siRNAs (Sigma-Aldrich) used were as follows: siPKM1 (SASI_Rn01_00106843), siPKM2 (SASI_Mm01_00036290), siFGF21 (SASI_Mm01_00050275), siCCND1 (SASI_Mm01_00041294), and siMYC (SASI_Mm01_00157475). The MISSION siRNA Universal Negative Control #1 (#SIC001) (siNEG) was used as control in all experiments.

RNA extraction and real-time quantitative PCR

Total RNA was purified using TRI Reagent (#T9424; Sigma-Aldrich) according to the instructions of the manufacturer, and reverse transcription and real-time quantitative PCR (RT–qPCR) were performed as described [22], except that SensiFAST SYBR Lo-ROX Kit (#BIO-94005; Bioline, London, UK) and RNaseOUT Recombinant Ribonuclease Inhibitor (#10777019; Life Technologies)

were used. Relative mRNA levels were determined by normalization to expression levels of TATA-binding protein (*Tbp*).

Primers used were as follows:

Ccnd1, fwd-GAGCTGCTGCAAATGGAAC, rev-CTCTGGCATTTTGGAGAGGA;

Cidea, fwd-AAAGGGACAGAAATGGACAC, rev-TTGAGACAGCCGAGGAAG;

Dio2, fwd-CAGTGTGGTGCACGTCTCCAATC, rev-TGAACCAAAGTTGACCACCAG;

Fgf-21, fwd-AGATGGAGCTCTCTATGGATCG, rev-GGGCTTCAGACTGGTACACAT;

Glut1, fwd-GGACCCTGCACCTCATTG, rev-GCCACGATGCTCAGATAGG;

Ldha, fwd-TAATGAAGGACTTGGCGGAT, rev-TTGGAGTTCGCAGTTACACA;

Mek5, fwd-CAACGGCCAGATGAATGAA, rev-AAGGATTTCCAGCTCAGACA;

Myc, fwd-TAGTGCTGCCATGAGGAGACAC, rev-CCACAGACACCACATCAATTT;

Pklr, fwd-GAGTCTTCCCCTTGCTCTACC, rev-CCTGTCACCAATCACCA;

Pkm1, fwd-CTGCTGTTTGAAGAGCTTGTG, rev-GAGTCACGGCAATGATAGGA;

Pkm2, fwd-TGCTGCAGTGGGGCCATTAT, rev-GAGTCACGGCAATGATAGGA;

Ppargc1a, fwd-AGCCGTGACCACTGACAACG-AG, rev-GCTGCATGGTTCTGAGTGCTAAG;

Tbp [23], fwd-ACCCTTCACCAATGACTCCTATG, rev-ATGATGACTGCAGCAAATCGC; and.

Ucp1, fwd-AGCCGGCTTAATGACTGGAG, rev-TCTGTAGGCTGCCCAATGAAC.

Whole-cell extracts and immunoblotting

Whole-cell extracts were prepared as described [24]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and transcription factor II B (TFIIB) were both used as loading controls in Fig. 1B, as we were unable to identify a loading control with stable levels between tissues. Primary antibodies used were PKM1 (#SAB4200094; Sigma-Aldrich), PKM2 (#SAB4200095; Sigma-Aldrich), GAPDH (#Ab8245; Abcam, Cambridge, UK), UCP1 (#Ab10983; Abcam), and TFIIB (#sc-225; Santa Cruz Biotechnology, Heidelberg, Germany). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse (#P0447; Dako, Glostrup, Denmark) and anti-rabbit (#P0448; Dako).

FGF21 ELISA

Four days after transfection with siNEG, siPKM1, or siPKM2, the cell culture medium was collected and centrifuged at 10 000 r.p.m. for 3 min at 4 °C. FGF21 concentrations were determined as described [25].

Pyruvate kinase activity measurements

PK activity measurements were performed with the PK Activity Assay Kit (#MAK072; Sigma-Aldrich) according to the instructions of the manufacturer. Reverse siRNA transfections were performed in WT-1 adipocytes at day 6, and at day 10, cells were harvested in PK Assay Buffer. Colorimetric measurements were performed on an Envision multiplate reader (Perkin–Elmer) with absorbance at 570 nm.

Statistical analyses

For WT-1 cells, three wells were harvested for each condition in each independent experiment. For primary brown cultures, two wells were harvested for each condition in each independent experiment. Data represent mean of means of three to four independent experiments + standard error of the mean (SEM). Statistical significance in the RT–qPCR data was determined on log-transformed data. Statistical significance for RT–qPCR, medium, and enzyme activity measurements was determined through 95% confidence intervals as calculated by paired one-way analysis of variance (ANOVA) (Figs 2D,E and 3B) or two-way ANOVA with Dunnett's correction for multiple comparisons (Figs 2A,C,F, 3A and 4A,B).

Results

PKM2 is highly expressed in brown adipose tissue

We have previously shown that the expression of PKs is induced in BAT by cold exposure and by β-adrenergic stimulation in primary brown adipocytes and in the brown adipocyte cell line WT-1 [11]. To gain insight into the expression levels and differences in expression between the different isoenzymes of PK, we profiled the mRNA levels of *Pklr, Pkm1,* and *Pkm2* in different mouse tissues. *Pklr* was highly expressed in the small intestine and liver, and *Pkm1* mRNA was detected at the highest level in skeletal muscle (Fig. 1A). The *Pkm2* mRNA was detectable in all tissues but was most abundantly present in the small intestine and BAT. In BAT, the mRNA level of *Pkm2* was fivefold higher than *Pkm1* and nearly 1000-fold higher than *Pklr*. Thus, in BAT, *Pkm2* was the most highly expressed isoform. The same pattern was observed at the level of protein where PKM1 was present at the highest level in skeletal muscle, and PKM2 was most abundant in BAT (Fig. 1B).

Knockdown of PKM2 in brown adipocytes increases UCP1 and FGF21 levels

To study the possibly different roles of the two PKM isoforms, we performed knockdown experiments with

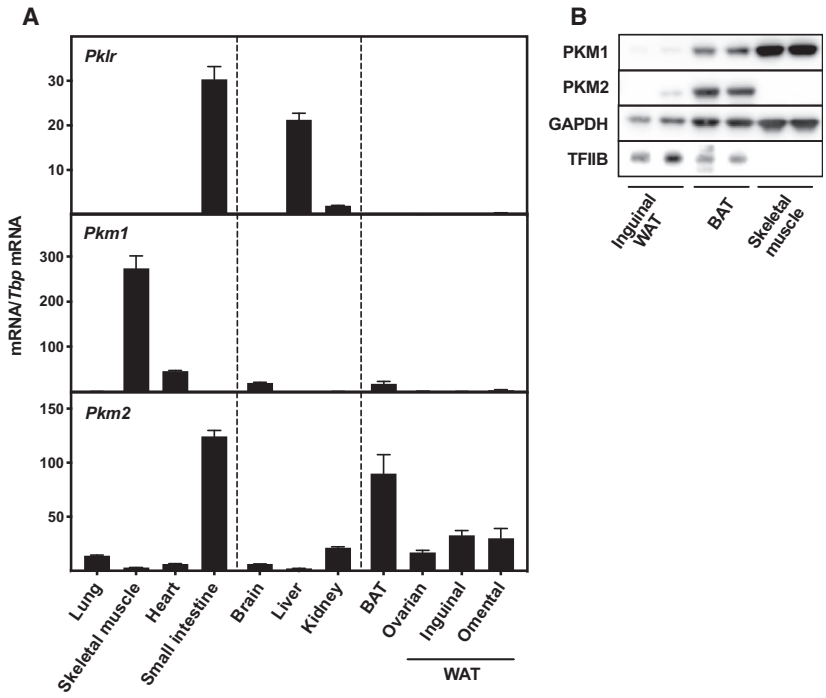

Fig. 1. PKM2 is highly expressed in BAT. (A) RT–qPCR profiling of *Pklr, Pkm1,* and *Pkm2* gene expression measured in eleven different mouse tissues (n = 5). *Tbp* mRNA levels were used for normalization. Data represent mean + SEM. (B) Immunoblotting analyses of PKM1 and PKM2 in inguinal WAT, BAT, and skeletal muscle. GAPDH and TFIIB were used as loading controls (n = 2).

siRNAs in mature WT-1 brown adipocytes [21]. The WT-1 brown preadipocytes were differentiated into mature adipocytes, transfected with control siRNA (universal negative siRNA, siNEG), siPKM1, or siPKM2, and harvested four days later. Knockdowns were verified at both mRNA (~ 95% for both *Pkm1* and *Pkm2*) and protein levels (Fig. 2A,B). We measured the expression of different thermogenic genes by RT–qPCR. Remarkably, knockdown of PKM2 led to significantly increased expression of *Ucp1* (eightfold), *Fgf21* (ninefold), and *Cidea* (twofold) compared with cells transfected with siNEG (Fig. 2C). No change was observed for two other thermogenic genes, *Ppargc1a* and *Dio2*. Correspondingly, we observed increased levels of UCP1 protein and secreted FGF21 in PKM2-depleted brown adipocytes compared with control and PKM1-depleted cells (Fig. 2B,D). Of notice, the increased expression of selected thermogenic genes was not accompanied by a decreased PK activity in PKM2-silenced adipocytes (Fig. 2E). However, knockdown of PKM1 resulted in a 40% decrease in PK activity (Fig. 2E).

To investigate whether the observations in the WT-1 cell line would be valid also for primary adipocyte cultures, we performed siRNA transfections with siNEG, siPKM1, or siPKM2 in mature spontaneously differentiated primary brown adipocytes. Expression of *Pkm1* and *Pkm2* was significantly reduced in response to transfection with siPKM1 or siPKM2, respectively, as determined by RT–qPCR (Fig. 2F). Knockdown of PKM2 in primary brown adipocytes, but not of PKM1, enhanced the expression of *Ucp1* and *Fgf21*. Taken together, PKM2 appears to regulate expression of certain thermogenesis-related genes such as *Ucp1* in brown adipocytes in a manner not linked to its PK activity.

FGF21 does not mediate the increased expression of *Ucp1* in response to knockdown of PKM2

FGF21 is a known regulator of thermogenic gene expression in adipose tissue [4]. Since the secretion of FGF21 was increased in PKM2-depleted cells, we investigated whether the action of PKM2 on *Ucp1* mRNA levels was mediated through FGF21. We performed knockdown experiments in WT-1 cells with siNEG, siPKM2, siFGF21, or a combination of the latter two. As above, knockdown of PKM2 led to a significantly increased expression of *Ucp1* and *Fgf21* (Fig. 3A). We did not obtain a significant knockdown of the *Fgf21* mRNA with siFGF21 alone (Fig. 3A), but a significant decrease in secreted FGF21 was observed (Fig. 3B). When combining siPKM2 and siFGF21, the induction of *Fgf21* expression obtained by PKM2 knockdown was attenuated and FGF21 secretion was decreased to below control values. Despite FGF21 secretion not being induced

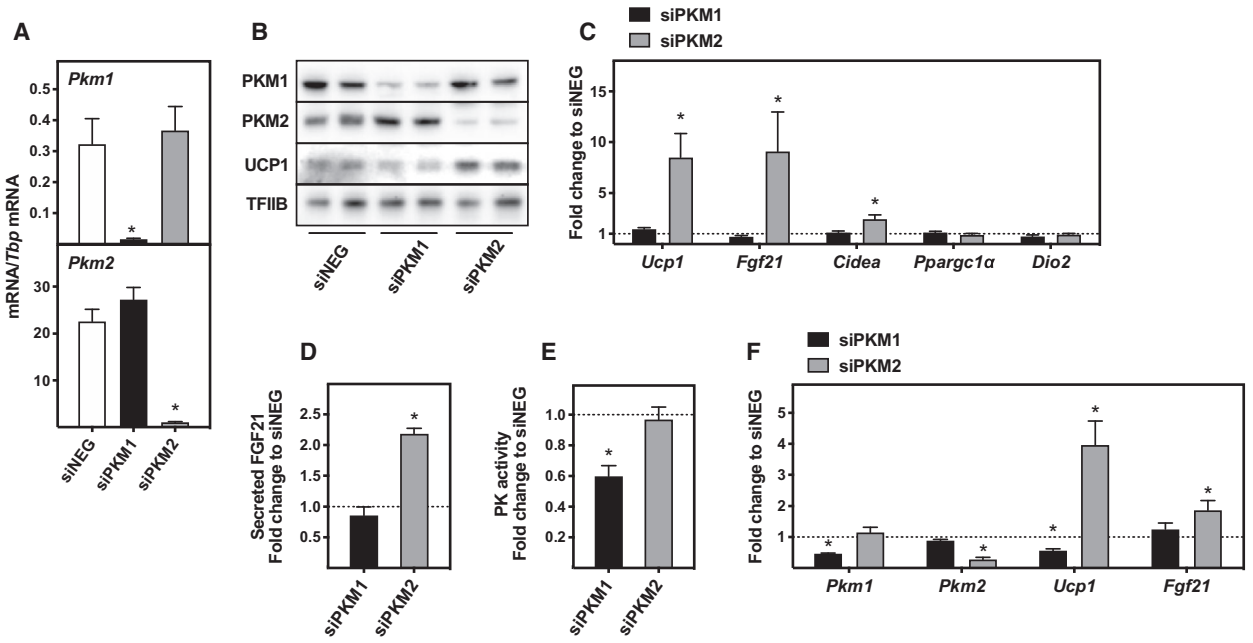

Fig. 2. Knockdown of PKM2 increases *Ucp1* and *Fgf21* expression in WT-1 brown adipocytes and primary brown adipocytes. (A–E) Reverse siRNA transfection of mature WT-1 adipocytes with siNEG, siPKM1, or siPKM2 was performed at day 6 of differentiation, and the cells were harvested 4 days later. (A) Relative mRNA levels of *Pkm1* and *Pkm2* were determined ($n = 4$). (B) Immunoblotting analyses of PKM1, PKM2, and UCP1 of WT-1 brown adipocytes harvested 4 days after siRNA transfection. TFIIB was used as loading control ($n = 2$). (C) Relative mRNA levels of *Ucp1*, *Fgf21*, *Cidea*, *Ppargc1a,* and *Dio2* were determined. The results are shown as fold change compared with siNEG ($n = 4$). (D) Fold difference in secreted FGF21 measured in the cell culture medium compared with siNEG ($n = 3$). (E) Fold difference in PK activity measured in PKM1- and PKM2-depleted adipocytes compared with siNEG ($n = 3$). (F) Reverse siRNA transfection of primary brown adipocytes with siNEG, siPKM1, or siPKM2 was performed 8 days after isolation, and the cells were harvested 4 days later. Relative mRNA levels of *Pkm1*, *Pkm2, Ucp1,* and *Fgf21* were determined and shown as fold change compared with siNEG ($n = 4$). Data represent mean of means + SEM. $^*P < 0.05$ versus siNEG.

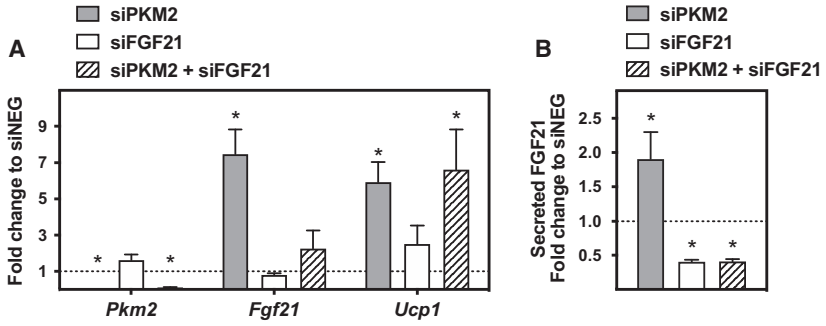

Fig. 3. The increased *Ucp1* expression in response to PKM2 knockdown is not mediated by FGF21 (A) Reverse siRNA transfection of mature WT-1 adipocytes with siNEG, siPKM2, siFGF21, or the combination of the latter two was performed at day 6 of differentiation, and the cells were harvested 4 days later. Relative mRNA levels of *Pkm2*, *Ucp1,* and *Fgf21* were determined and shown as fold change compared with siNEG. (B) Fold difference in secreted FGF21 measured in the cell culture medium compared with siNEG. Data represent mean of means + SEM ($n = 4$). $^*P < 0.05$ versus siNEG.

when combining siPKM2 and siFGF21, PKM2 knockdown still led to a significantly increased expression of *Ucp1*. These results indicate that the increase in *Ucp1* expression induced by PKM2 depletion is not mediated by increased FGF21 secretion to the medium.

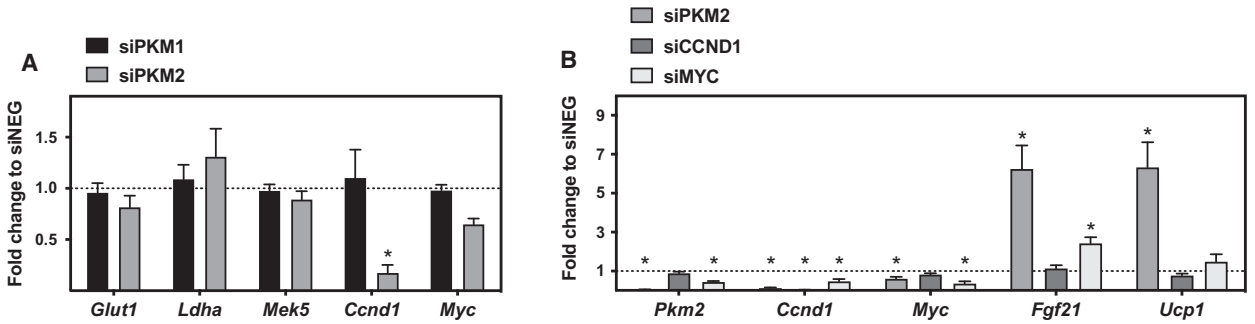

Fig. 4. The effect of PKM2 knockdown on thermogenic gene expression is not mediated by known PKM2-dependent transcriptional events. (A) Relative mRNA levels of *Glut1*, *Ldha*, *Mek5*, *Ccnd1*, and *Myc* in cells transfected with siNEG, siPKM1, or siPKM2 were determined and shown as fold change compared with siNEG. (B) Reverse siRNA transfection of mature WT-1 adipocytes with siNEG, siPKM2, siCCND1, or siMYC was performed at day 6 of differentiation, and the cells were harvested 4 days later. Relative mRNA levels of *Pkm2*, *Ccnd1*, *Myc*, *Fgf21*, and *Ucp1* were determined and shown as fold change compared with siNEG. Data represent mean of means + SEM ($n = 4$). *$P < 0.05$ versus siNEG.

Cyclin D1 and MYC do not mediate the increased expression of *Ucp1* in response to knockdown of PKM2

As the effect of PKM2 knockdown was not mediated *via* secreted FGF21, we examined possible nuclear mediation of the effect. PKM2 contains an inducible nuclear localization signal, and in the nucleus, PKM2 can act as a cofactor for several transcription factors [17,18]. We therefore measured the expression of five known target genes of the three established transcriptional pathways downstream of PKM2. One of the main transcriptional pathways downstream of PKM2 is the hypoxia-inducible factor 1α pathway in which PKM2 acts as a coactivator to increase expression of genes such as glucose transporter 1 (*Glut1*) and lactate dehydrogenase A (*Ldha*) [26]. If the effect of PKM2 on *Ucp1* and *Fgf21* gene expression was mediated *via* the HIF-1α pathway, the PKM2-HIF-1α module would be predicted to exert a negative effect on transcription of the *Ucp1* and *Fgf21* genes, and in addition, it would be expected that *Glut1* and *Ldha* would be decreased by PKM2 knockdown. However, knockdown of PKM2 had no impact on expression of *Glut1* and *Ldha* in WT-1 adipocytes (Fig. 4A). Therefore, the HIF-1α pathway is not likely to be involved in the PKM2-mediated negative effect on *Ucp1* and *Fgf21* mRNA levels.

PKM2 can also activate transcription of the mitogen-activated protein kinase kinase 5 (*Mek5*) gene by phosphorylating the signal transducer and activator of transcription 3 [19]. However, knockdown of PKM2 in WT-1 brown adipocytes had no impact on expression of *Mek5* (Fig. 3A). This indicates that the MEK5-STAT pathway is not involved in the PKM2-dependent inhibition of *Ucp1* and *Fgf21* gene expression.

Thirdly, in other cell systems, a complex of PKM2 and β-catenin (CTNNB1) has been shown to promote transcription of the cyclin D1 (*Ccnd1*) and v-myc avian myelocytomatosis viral oncogene homolog (*Myc*) genes [18,27]. Knockdown of PKM2 in WT-1 adipocytes, but not PKM1, decreased the expression of *Ccnd1* and tended to decrease the expression of *Myc* (Fig. 4A). To examine whether the decreased expression of *Ccnd1* or *Myc* could explain the PKM2 effect on thermogenic gene expression, we examined the effect of knocking down CCND1 or MYC (or PKM2) in mature WT-1 adipocytes (Fig. 4B). Transfection with siCCND1 had a significant effect on the mRNA level of *Ccnd1,* but this was not associated with any effect on *Ucp1* or *Fgf21* gene expression. Similarly, transfection with siMYC led to a significant reduction in the mRNA levels of *Myc, Pkm2,* and *Ccnd1*, which is consistent with both *Pkm2* and *Ccnd1* being known target genes of MYC [28,29]. MYC depletion led to a modest, yet significant, increase in *Fgf21* expression, but had no effect on *Ucp1* expression.

Since we observed that knockdown of PKM2 caused a downregulation of *Ccnd1* and *Myc* expression, it suggests that the PKM2–CTNNB1 complex is operational in brown adipocytes. However, knockdown of CCND1 and MYC had in itself no influence on *Ucp1* expression, and this pathway is therefore not likely to be involved in the PKM2-mediated decrease in *Ucp1* gene expression.

Discussion

PKM2 is known for its role in cancer cell biology [30,31]. The increased expression of PK in BAT induced by cold [11] suggests a function of PKM2 also in adipose tissue. Here, we report that PKM2 is expressed at higher levels in BAT compared with white adipose tissue (WAT) (Fig. 1). Particularly, we show that in mature brown adipocytes, PKM2 depletion has an unexpected and potentially important positive effect

on expression of the thermogenesis-related genes *Ucp1* and *Fgf21*, and this effect appears to be independent on PKM2's PK activity (Fig. 2).

In the present study, we have experimentally examined whether the effect of PKM2 depletion on thermogenic gene expression was mediated through one of the established nuclear pathways, that is, the HIF-1α pathway, the MEK5 pathway, and the CTNNB1 pathway. We found no indications that these pathways were involved.

PKM2 interacts with the tumor suppressor p53 and negatively modulates its transcriptional activity [17,32]. Therefore, by knocking down PKM2 we would expect a higher activity of p53, but as p53-deficient mice and adipocytes displayed increased expression of *Ucp1* [33], and as WT-1 cells have compromised p53 function due to expression of simian virus 40 large T antigen [34], we do not consider p53 a potential mediator of PKM2 function in our studies. Thus, the effect of PKM2 on UCP1 levels does not appear to be mediated through known interaction partners of PKM2. This conclusion evidently points to further studies dedicated to identification of the novel molecular mechanism involved in this effect.

However, physiologically and translationally, our findings demonstrate an important role of the multifunctional glycolytic enzyme PKM2 in the regulation of the expression of key thermogenesis-related genes in brown adipocytes. Whether the expression level of PKM2 has a physiological regulatory role in the regulation of the expression of these genes still needs to be investigated. However, irrespective of this, our studies imply that targeting PKM2 activity in adipocytes could have beneficial effects on energy expenditure, with a potential beneficial impact on systemic metabolism. This improved understanding of the regulation of thermogenic gene expression and thereby the thermogenic capacity of adipose tissue may thus provide new avenues for potential therapeutic interventions in metabolic dysfunction.

Acknowledgements

We thank Lillian H. L. Hansen, Amanda Cheung, and Patricia S. S. Petersen for technical assistance.

Author contributions

MSI, SW, LKM, ALB, BQ, JN, BE, and JBH conceived and designed the experiments. MSI, SW, LKM, and ALB performed the experiments. MSI, SW, and JBH analyzed and interpreted the data. MSI prepared the figures. MSI and JBH wrote the paper. All authors read and approved the final manuscript.

Funding

This work was supported by grants to JBH from the Novo Nordisk Foundation and the EU FP7 project DIABAT (HEALTH-F2-2011-278373) and to JN from the Swedish Science Research Council. MSI and BE were supported by the Novo Nordisk Foundation. The Novo Nordisk Foundation Center for Basic Metabolic Research is an independent research center at the University of Copenhagen partially funded by an unrestricted donation from the Novo Nordisk Foundation.

References

1 Nedergaard J, Bengtsson T and Cannon B (2007) Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* **293**, E444–E452.

2 Cannon B and Nedergaard J (2004) Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277–359.

3 Feldmann HM, Golozoubova V, Cannon B and Nedergaard J (2009) UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* **9**, 203–209.

4 Fisher FM,Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, Wu J, Kharitonenkov A, Flier JS, Maratos-Flier E *et al.* (2012) FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* **26**, 271–281.

5 de Jong JM, Larsson O, Cannon B and Nedergaard J (2015) A stringent validation of mouse adipose tissue identity markers. *Am J Physiol Endocrinol Metab* **308**, E1085–E1105.

6 Hondares E, Iglesias R, Giralt A, Gonzalez FJ, Giralt M, Mampel T and Villarroya F (2011) Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J Biol Chem* **286**, 12983–12990.

7 de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, Larsen PR and Bianco AC (2001) The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. *J Clin Invest* **108**, 1379–1385.

8 Barbera MJ, Schluter A, Pedraza N, Iglesias R, Villarroya F and Giralt M (2001) Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. *J Biol Chem* **276**, 1486–1493.

9 Lee P, Smith S, Linderman J, Courville AB, Brychta RJ, Dieckmann W, Werner CD, Chen KY and Celi FS (2014) Temperature-acclimated brown adipose tissue

modulates insulin sensitivity in humans. *Diabetes* **63**, 3686–3698.
10 Hao Q, Yadav R, Basse AL, Petersen S, Sonne SB, Rasmussen S, Zhu Q, Lu Z, Wang J, Audouze K *et al.* (2015) Transcriptome profiling of brown adipose tissue during cold exposure reveals extensive regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* **308**, E380–E392.
11 Basse AL, Isidor MS, Winther S, Skjoldborg NB, Murholm M, Andersen ES, Pedersen SB, Wolfrum C, Quistorff B and Hansen JB (2017) Regulation of glycolysis in brown adipocytes by HIF-1alpha. *Sci Rep* **7**, 4052.
12 Noguchi T, Yamada K, Inoue H, Matsuda T and Tanaka T (1987) The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem* **262**, 14366–14371.
13 Noguchi T, Inoue H and Tanaka T (1986) The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J Biol Chem* **261**, 13807–13812.
14 Imamura K and Tanaka T (1972) Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. I. Electrophoretic studies. *J Biochem* **71**, 1043–1051.
15 Yang W and Lu Z (2013) Regulation and function of pyruvate kinase M2 in cancer. *Cancer Lett* **339**, 153–158.
16 Dayton TL, Jacks T and Vander Heiden MG (2016) PKM2, cancer metabolism, and the road ahead. *EMBO Rep* **17**, 1721–1730.
17 Xia L, Wang XR, Wang XL, Liu SH, Ding XW, Chen GQ and Lu Y (2016) A novel role for pyruvate kinase M2 as a corepressor for P53 during the DNA damage response in human tumor cells. *J Biol Chem* **291**, 26138–26150.
18 Yang W, Xia Y, Hawke D, Li X, Liang J, Xing D, Aldape K, Hunter T, Alfred Yung WK and Lu Z (2012) PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* **150**, 685–696.
19 Gao X, Wang H, Yang JJ, Liu X and Liu ZR (2012) Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol Cell* **45**, 598–609.
20 Fasshauer M, Klein J, Ueki K, Kriauciunas KM, Benito M, White MF and Kahn CR (2000) Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. *J Biol Chem* **275**, 25494–25501.
21 Isidor MS, Winther S, Basse AL, Petersen MC, Cannon B, Nedergaard J and Hansen JB (2016) An siRNA-based method for efficient silencing of gene expression in mature brown adipocytes. *Adipocyte* **5**, 175–185.
22 Murholm M, Dixen K, Qvortrup K, Hansen LH, Amri EZ, Madsen L, Barbatelli G, Quistorff B and Hansen JB (2009) Dynamic regulation of genes involved in mitochondrial DNA replication and transcription during mouse brown fat cell differentiation and recruitment. *PLoS ONE* **4**, e8458.
23 Hansen JB, Petersen RK, Larsen BM, Bartkova J, Alsner J and Kristiansen K (1999) Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. *J Biol Chem* **274**, 2386–2393.
24 Winther S, Isidor MS, Basse AL, Skjoldborg N, Cheung A, Quistorff B and Hansen JB (2018) Restricting glycolysis impairs brown adipocyte glucose and oxygen consumption. *Am J Physiol Endocrinol Metab* **314**, E214–E223.
25 Markussen LK, Winther S, Wicksteed B and Hansen JB (2018) GSK3 is a negative regulator of the thermogenic program in brown adipocytes. *Sci Rep* **8**, 3469.
26 Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, Cole RN, Pandey A and Semenza GL (2011) Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* **145**, 732–744.
27 Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, Gao X, Aldape K and Lu Z (2011) Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. *Nature* **480**, 118–122.
28 David CJ, Chen M, Assanah M, Canoll P and Manley JL (2010) HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* **463**, 364–368.
29 Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, Cocito A and Amati B (2003) Genomic targets of the human c-Myc protein. *Genes Dev* **17**, 1115–1129.
30 Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL and Cantley LC (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230–3.
31 Spoden GA, Mazurek S, Morandell D, Bacher N, Ausserlechner MJ, Jansen-Durr P, Eigenbrodt E and Zwerschke W (2008) Isotype-specific inhibitors of the glycolytic key regulator pyruvate kinase subtype M2 moderately decelerate tumor cell proliferation. *Int J Cancer* **123**, 312–321.
32 Wu H, Yang P, Hu W, Wang Y, Lu Y, Zhang L, Fan Y, Xiao H and Li Z (2016) Overexpression of PKM2 promotes mitochondrial fusion through attenuated p53 stability. *Oncotarget* **7**, 78069–78082.
33 Hallenborg P, Fjære E, Liaset B, Petersen RK, Murano I, Sonne SB, Falkerslev M, Winther S, Jensen BA, Ma T *et al.* (2016) p53 regulates expression of uncoupling protein 1 through binding and repression of PPARgamma coactivator-1alpha. *Am J Physiol Endocrinol Metab* **310**, E116–E128.
34 Ali SH and DeCaprio JA (2001) Cellular transformation by SV40 large T antigen: interaction with host proteins. *Semin Cancer Biol* **11**, 15–23.