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An siRNA-based method for efficient silencing of gene expression in mature brown adipocytes

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ABSTRACT

Brown adipose tissue is a promising therapeutic target for opposing obesity, glucose intolerance and insulin resistance. The ability to modulate gene expression in mature brown adipocytes is important to understand brown adipocyte function and delineate novel regulatory mechanisms of non-shivering thermogenesis. The aim of this study was to optimize a lipofection-based small interfering RNA (siRNA) transfection protocol for efficient silencing of gene expression in mature brown adipocytes. We determined that a critical parameter was to deliver the siRNA to mature adipocytes by reverse transfection, i.e. transfection of non-adherent cells. Using this protocol, we effectively knocked down both high- and low-abundance transcripts in a model of mature brown adipocytes (WT-1) as well as in primary mature mouse brown adipocytes. A functional consequence of the knockdown was confirmed by an attenuated increase in uncoupled respiration (thermogenesis) in response to β-adrenergic stimulation of mature WT-1 brown adipocytes transfected with uncoupling protein 1 siRNA. Efficient gene silencing was also obtained in various mouse and human white adipocyte models (3T3-L1, primary mouse white adipocytes, hMADS) with the ability to undergo "browning." In summary, we report an easy and versatile reverse siRNA transfection protocol to achieve specific silencing of gene expression in various models of mature brown and browning-competent white adipocytes, including primary cells.

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Introduction

Brown adipose tissue (BAT) is specialized in non-shivering thermogenesis, by which energy is used for the generation of heat.[1-4] The protein responsible for this process is uncoupling protein 1 (UCP1), located in the inner mitochondrial membrane, where it uncouples the electron transport chain from ATP production. BAT activity is important for rodents to defend their body temperature during prolonged cold exposure and has been shown to counteract many of the harmful effects of a high-fat diet, such as obesity, glucose intolerance and insulin resistance.[1-4] Evidence from rodent studies demonstrates that BAT plays a significant role in glucose and lipid metabolism as well as in whole-body energy homeostasis. The observations that adult humans possess metabolically active BAT, that BAT in humans is recruitable and that BAT mass influences whole-body energy homeostasis make brown adipocytes an interesting target in combatting metabolic disease.[5,6]

Probing the involvement of selected genes in brown adipocyte function can be achieved through loss- and gain-of-function approaches. Generation of transgenic or gene knockout organisms has been of crucial importance for deciphering BAT development and function, but it is both time-consuming and expensive, and moreover, this approach is limited to rodent models. Therefore, an efficient method to silence gene expression in cultured mature brown adipocytes is a relevant supplement to genetically modified mice or rats. RNA interference (RNAi) is a powerful tool to achieve gene silencing; however, using RNAi in mature adipocytes is challenging due to the ineffective delivery of siRNA and short-hairpin RNA (shRNA) by non-viral and viral means, respectively. Using RNAi in pre-adipocytes is straightforward,

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but is of limited use if the aim is to understand the function of the targeted gene in the mature adipocyte state: silencing of gene expression by RNAi (by means of siRNA delivery or stable viral expression of shRNA) may affect differentiation *per se*, and even if differentiation is unaffected, gene silencing by siRNA delivery is transient and is not likely to last until the mature adipocyte state is reached. It is possible to efficiently transduce mature adipocytes with adeno- or lentiviral vectors, however, such an approach is relatively labor intensive and requires special safety precautions.[7,8] Of non-viral approaches, electroporation has been used for delivery of siRNA into fully differentiated adipocytes;[9-12] however, this strategy requires large amounts of siRNA and/or results in a poor transfection and knockdown efficiency. Recently, significant advances have been made by using lipid-based transfection reagents for siRNA transfection of mature white adipocytes.[13-15] To our knowledge, no studies have reported efficient siRNA-based knockdown of gene expression in mature brown adipocytes. Therefore, based on the protocol reported by Kilroy et al.,[13] we set out to develop a protocol for efficient gene silencing in mature brown adipocytes.

We here report an easy and versatile reverse siRNA transfection protocol to silence specific gene expression in mature brown adipocytes and in mature white adipocytes capable of undergoing "browning." Importantly, the protocol works well in human adipocytes as well as in primary mouse adipocytes, and it can be used in combination with downstream analyses such as studies of insulin signaling, lipolysis and thermogenesis.

Results

Efficient silencing of gene expression in mature mouse brown adipocytes by reverse siRNA transfection

In order to silence specific genes in mature brown adipocytes without interfering with adipogenesis, we set out to optimize a lipofection-based siRNA transfection protocol. We based our optimization on a protocol developed by Kilroy et al. to obtain effective silencing of gene expression in white adipocytes.[13] Using the Lipofectamine RNAiMAX Transfection Reagent, we probed the importance of experimental parameters such as siRNA concentration and incubation time. For various immortalized cell models, we found that the optimal final concentration of siRNA was 50 nM and the optimal incubation time of the siRNA/RNAiMAX mix was 25 min. As described below, optimal conditions for primary mouse adipocytes were slightly different. In 96-well format, we compared siRNA transfection of adherent (forward transfection) and non-adherent (reverse transfection) pre-adipocytes and adipocytes. An overview of the reverse transfection workflow is shown in Figure 1. We used an siRNA targeting pyruvate kinase M (*Pkm*) and a non-silencing (negative) siRNA as control. Forward transfection of immortalized WT-1 brown pre-adipocytes and mature adipocytes with siRNA resulted in a similar *Pkm* knockdown efficiency of ~55% as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. 2A and B). Reverse transfection with *Pkm* siRNA yielded a 74% and 93% knockdown in pre-adipocytes and mature adipocytes, respectively (Fig. 2A and B). Thus, the reverse transfection protocol proved overall to be most efficient, particularly in the mature WT-1 brown adipocytes.

To render probable that the reverse transfected cells were primarily fully differentiated adipocytes, we confirmed that mature WT-1 adipocytes were able to attach after replating. Micrographs of an oil red O-stained 10-cm dish before replating and 48-well after replating showed that most of the adherent cells stained positive, demonstrating that mature adipocytes were able to re-attach after replating (Fig. 2C).

In accordance with the decrease in *Pkm* mRNA levels, transfection with *Pkm* siRNA resulted in a substantial decrease in PKM protein levels (Fig. 3A and B). To verify that the knockdown did not affect basic adipocyte function or affected the maturity of the adipocytes, we evaluated the effect of *Pkm* siRNA transfection on selected parameters. Knockdown of *Pkm* did not affect expression of typical adipocyte-enriched genes, such as fatty acid-binding protein 4 (*Fabp4*) and glucose transporter 4 (*Glut4*) (Fig. 3C and 3D), suggesting that the ratio of mature adipocytes to undifferentiated cells that re-attached after reverse transfection was similar irrespective of the siRNA used. Stimulation with insulin demonstrated that insulin signaling, measured by Ser473 phosphorylation of AKT, was not influenced by *Pkm* siRNA compared to negative control siRNA (Fig. 3B). Similarly, *Pkm* knockdown had no effect on adrenergically induced lipolysis, since a 6–7-fold increase in glycerol release after stimulation with the β-adrenergic agonist isoproterenol (ISO) was observed both in control and *Pkm* siRNA-transfected cells (Fig. 3E).

In summary, WT-1 brown adipocytes reverse transfected with siRNA displayed a normal response to insulin and β-adrenergic stimulation.

Efficient knockdown irrespective of target mRNA abundance

Pkm is expressed at relatively high levels in mature brown adipocytes (C_t value ~20). To compare the knockdown

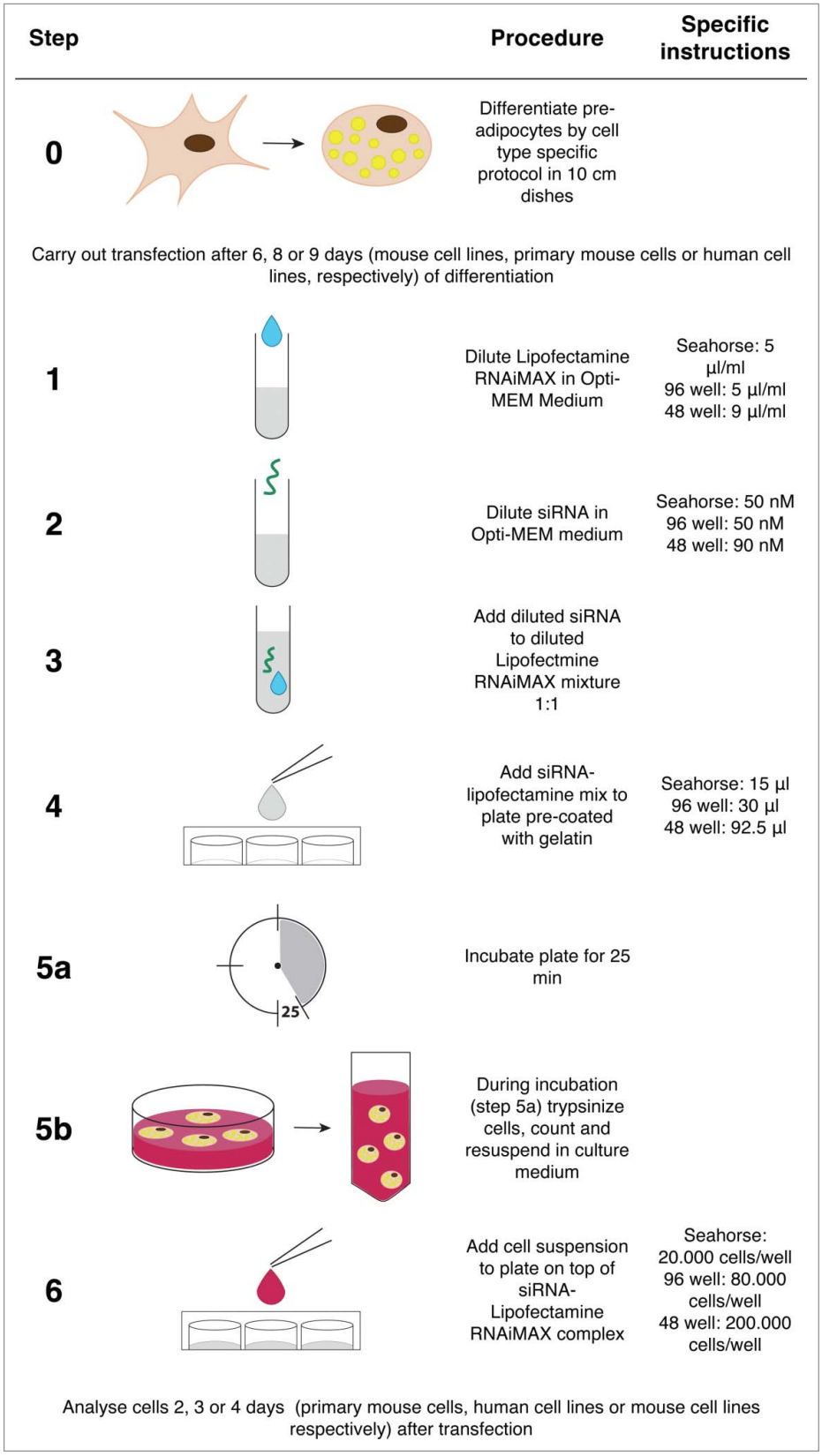

Figure 1. Schematic overview of the reverse siRNA transfection protocol.

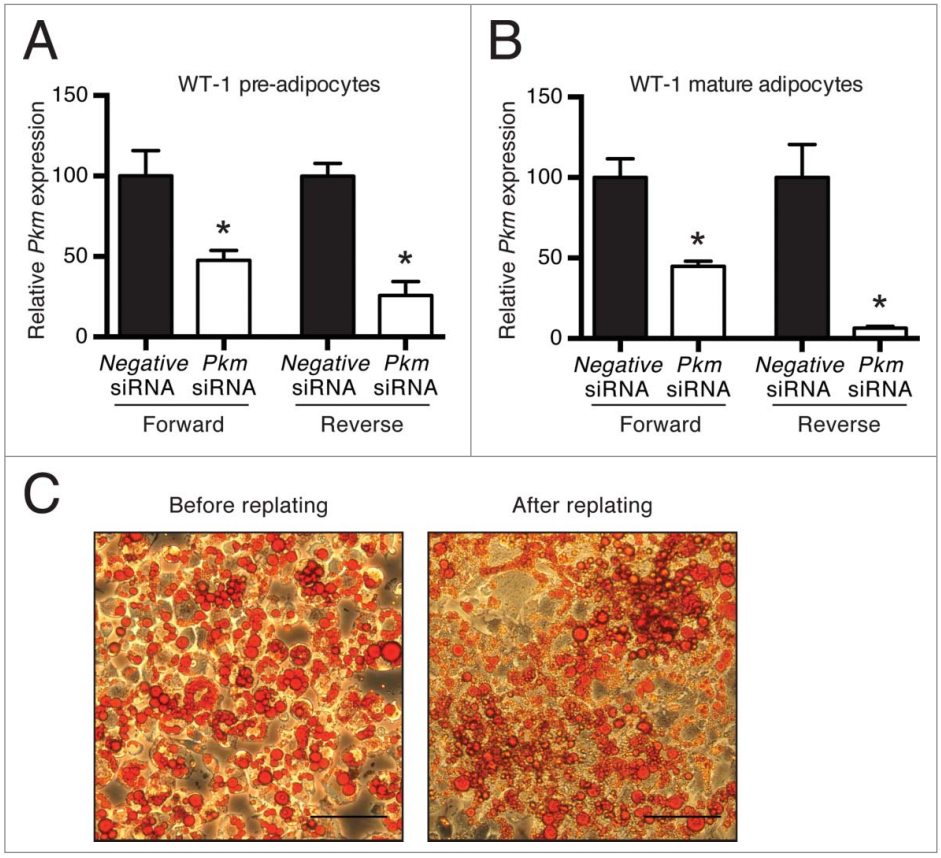

Figure 2. Comparison of *Pkm* knockdown efficiency in pre-adipocytes and adipocytes using forward and reverse siRNA transfection. Forward and reverse siRNA transfections of WT-1 pre-adipocytes and mature adipocytes with an siRNA against *Pkm* or a universal negative siRNA as control. The transfections were performed when the cells were ~70% confluent for the pre-adipocytes or at day 6 of differentiation for the mature adipocytes. The pre-adipocytes were harvested 2 d after transfection, and the mature adipocytes were harvested 4 d after transfection. Relative mRNA expression (measured by RT-qPCR) of *Pkm* was determined by normalization to expression levels of TATA-binding protein (*Tbp*). (A) Forward and reverse siRNA transfection of WT-1 pre-adipocytes. (B) Forward and reverse siRNA transfection of mature WT-1 adipocytes. Data represent mean of means +SEM ($n = 3$). *, $p < 0.05$ versus universal negative siRNA. (C) Oil red O staining of mature WT-1 adipocytes before and 4 d after replating. The scale bar equals 100 μm.

efficiency of high- and low-abundance mRNAs using reverse transfection of mature WT-1 adipocytes, we applied siRNA targeting *Pkm* or calcium/calmodulin-dependent protein kinase IIα (*Camk2a*), the latter being expressed at relatively low levels as determined by RT-qPCR (C_t value ~27). *Pkm* mRNA was knocked down by 89% upon transfection with *Pkm* siRNA (Fig. 4A), whereas *Camk2a* mRNA was reduced by 82% in response to *Camk2a* siRNA (Fig. 4B). Hence, both high- and low-abundance mRNAs were efficiently silenced in mature brown adipocytes by reverse siRNA transfection. Expression of *Fabp4* and *Glut4* mRNAs was not differentially influenced by the siRNAs (Fig. 4C and 4D).

Silencing of gene expression in human multipotent adipose-derived stem cell (hMADS), in the 3T3-L1 cell line and in primary mouse adipocytes

We further analyzed the efficiency and applicability of the reverse transfection protocol in 4 additional adipocyte cell models of human and mouse origin: hMADS, 3T3-L1 and primary mouse adipocytes from BAT and the inguinal white adipose tissue (iWAT) (Fig. 5). hMADS, 3T3-L1 and primary iWAT adipocytes are considered models of white adipogenesis, but all have the ability under specific conditions to convert into UCP1-positive, thermogenic adipocytes, a process known as "browning."[16-18] Reverse transfection of hMADS and 3T3-L1 adipocytes was carried out as for WT-1 adipocytes (Fig. 1). In order to obtain sufficient material for downstream analyses, primary mouse adipocytes were reverse transfected in 48-well plates, using 2.5-fold more cells per well and 90 nM siRNA (Fig. 1). We used siRNA against glyceraldehyde-3-phosphate (*GAPDH*) in the human hMADS adipocytes and *Pkm* siRNA in the 3 mouse adipocyte models. *GAPDH* mRNA was knocked down by 90% in hMADS adipocytes, and knockdown efficiencies of *Pkm* mRNA in 3T3-L1 and primary BAT and iWAT adipocytes were 90%, 73% and 80%, respectively (Fig. 5A-D). Hence, the

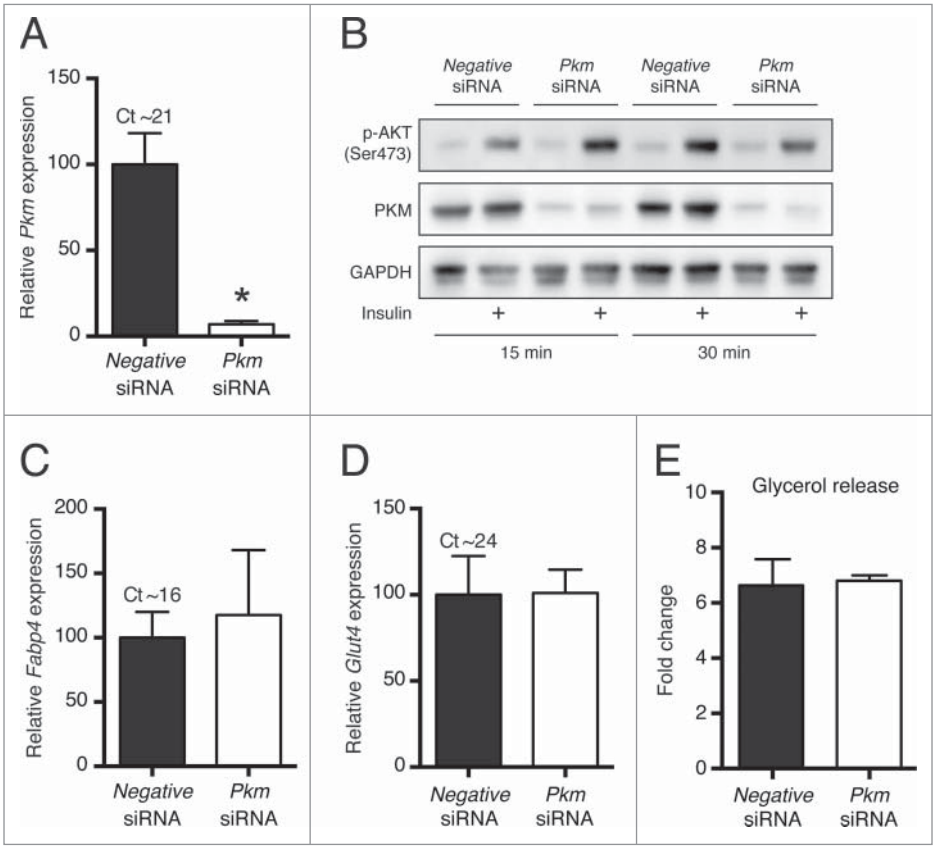

Figure 3. Effect of reverse siRNA transfection on brown adipocyte function. Mature WT-1 adipocytes reverse transfected with *Pkm* siRNA or universal negative siRNA at day 6 of adipocyte differentiation and harvested 4 d later. Relative mRNA expression levels (measured by RT-qPCR) of (A) *Pkm*, (C) *Fabp4, and* (D) *Glut4* were determined by normalization to expression levels of *Tbp*. Data represent mean of means +SEM (n = 3). *, $p < 0.05$ vs. universal negative siRNA. (B) Immunoblotting analyses of Ser473-phosphorylated AKT (p-AKT) and PKM following stimulation with 5 μg/ml insulin for 15 or 30 min. GAPDH was used as a loading control (n = 2). (E) Cell culture medium glycerol content from reverse transfected cells stimulated with 1 μM isoproterenol for 6 h, depicted as fold increase compared to non-stimulated cells. Data represent mean of means +SEM (n = 3).

optimized protocol was also applicable to human adipocytes (hMADS), mouse white adipocytes (3T3-L1 and primary iWAT) and primary mouse brown adipocytes.

Attenuation of β-adrenergically stimulated oxygen consumption in mouse brown adipocytes by Ucp1 knockdown

To demonstrate a functional consequence of gene silencing in brown adipocytes, we reverse transfected mature WT-1 adipocytes with an siRNA against *Ucp1*, which resulted in a 78% decrease in *Ucp1* mRNA levels, or with a control siRNA (Fig. 6A). Since *Ucp1* is barely expressed in pre-adipocytes, the *Ucp1* knockdown demonstrated that mature adipocytes had been transfected. To investigate the efficiency of the UCP1 knockdown on adrenergically induced thermogenesis, we performed the reverse transfection with *Ucp1* siRNA directly in gelatin-coated XF96 Cell Culture Microplates, to be used for analysis on the Seahorse XF analyzer. Four days after transfection, we determined oxygen consumption rate (OCR) during sequential addition of ISO, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) and rotenone/antimycin A (Fig. 6B). This was carried out under experimental conditions where it has been demonstrated that adrenergically induced OCR is UCP1-dependent.[19] Brown adipocytes transfected with the control siRNA increased their OCR by 70% in response to acute stimulation with ISO, whereas *Ucp1* siRNA-transfected cells increased their OCR by only 30% (Fig. 6B and C), demonstrating both the functional efficiency of the knockdown and the significance of UCP1 for the induced thermogenesis. *Ucp1* knockdown cells pretreated with oligomycin failed to increase oxygen consumption in response to ISO, whereas OCR increased with 30% in oligomycin-pretreated control cells (data not shown). Similar to the situation in freshly isolated mouse brown adipocytes,[20] this demonstrates that UCP1 is essential for adrenergically induced thermogenesis in mature WT-1 cells.

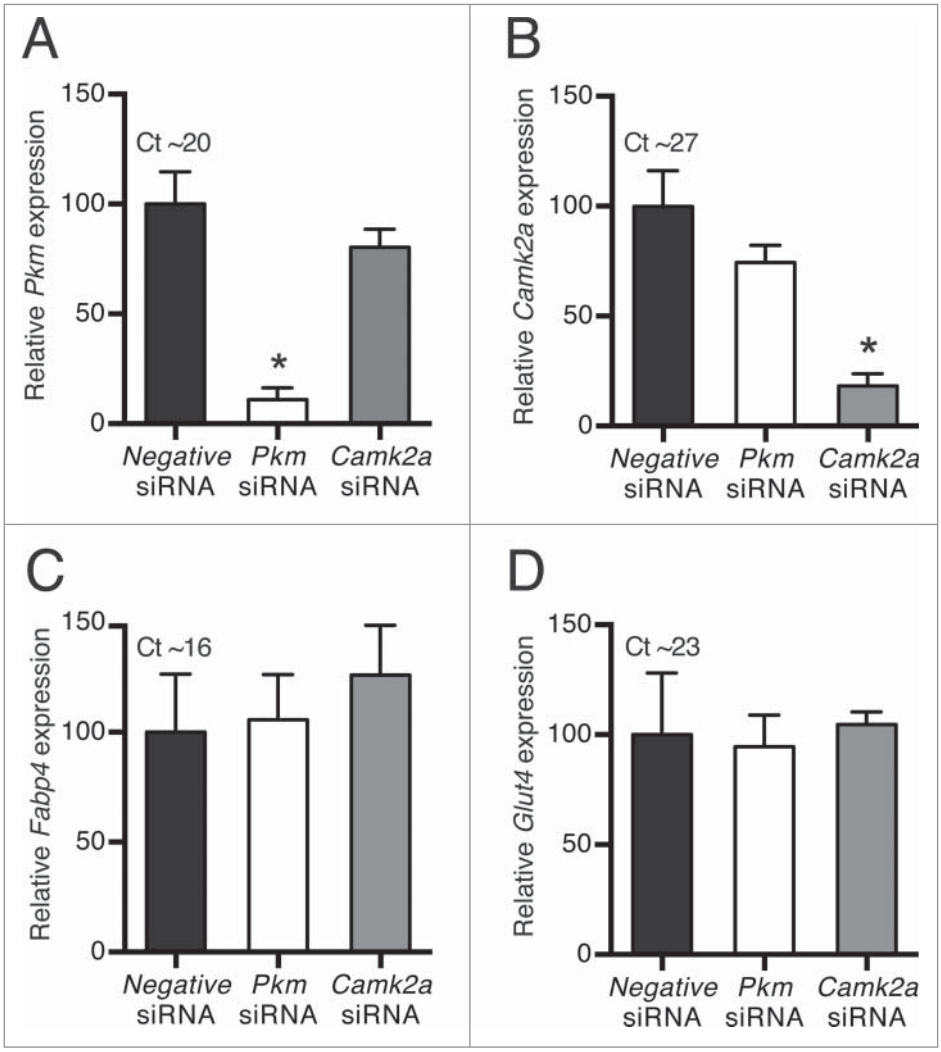

Figure 4. Knockdown efficiency of high- and low-abundance targets. Reverse siRNA transfections of mature WT-1 adipocytes with siRNA against *Pkm, Camk2a* or a universal negative siRNA as control. The transfections were performed at day 6 of differentiation and adipocytes were harvested 4 d later. Relative mRNA expression levels (RT-qPCR) of (A) *Pkm*, (B) *Camk2a*, (C) *Fabp4*, and (D) *Glut4* were determined by normalization to expression levels of *Tbp*. Data represent mean of means +SEM (n = 4). *, $p < 0.05$ versus universal negative siRNA.

Discussion

Brown adipocytes are considered promising targets in combatting obesity and diabetes. The ability to tinker with specific genes in mature brown adipocytes is of importance for discovering novel information about brown adipocyte biology. Here we report an easy, rapid and versatile lipofection-based reverse siRNA transfection protocol with which specific genes can be effectively silenced in mature adipocytes of various origins.

Transfecting the mature adipocytes while still in suspension, followed by reattachment, greatly increases knockdown efficiency. This reverse transfection protocol is effective using pre-adipocytes, but surprisingly is more effective using mature adipocytes. The presented protocol, as summarized in Figure 1, has an easy workflow, is time and cost efficient and requires no special equipment. The protocol is easily adaptable to various adipocyte cell models, as demonstrated by its effectiveness in different mouse and human adipogenic cell lines as well as in primary mouse adipocytes. Equally efficient gene silencing was observed in mature brown adipocytes and mature white adipocytes with the ability to undergo browning.

We also demonstrate that the knockdown procedure can be combined with functional assays, as shown by the normal response to insulin and β-adrenergic stimulation after reverse siRNA transfection, and the reduced β-adrenergically induced oxygen consumption following knockdown of *Ucp1* expression. Finally, the reverse siRNA transfection protocol has been optimized and standardized for 96-well plates and is therefore adaptable to high-throughput screening of siRNA libraries.

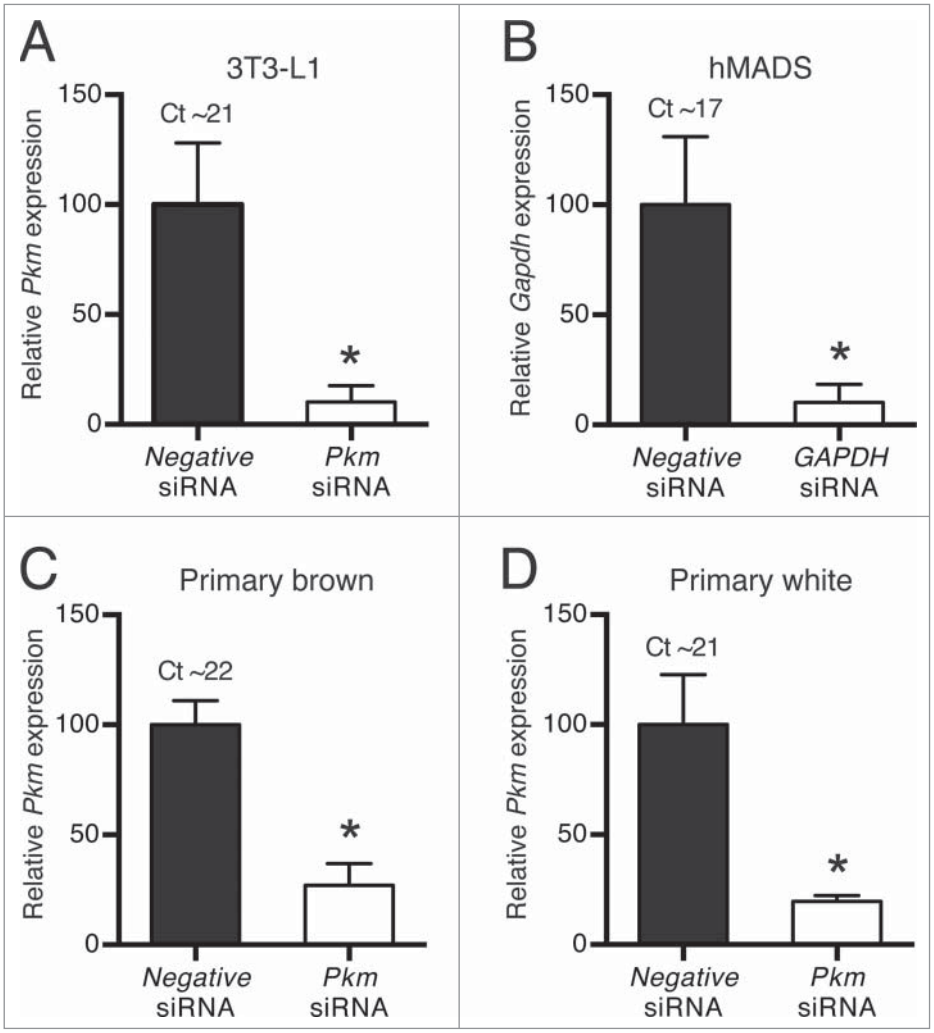

Figure 5. Knockdown efficiency in different adipocyte cell models. Reverse siRNA transfections of mature adipocytes with siRNA against *Pkm* for mouse cell models, *GAPDH* for human hMADS cells or a universal negative siRNA as control. The transfections were performed in (A) 3T3-L1, (B) hMADS, and (C) primary brown and (D) primary white adipocytes. The reverse siRNA transfections were performed at day 6 (3T3-L1), day 9 (hMADS) or day 8 (primary cells) of differentiation and were harvested 4 d, 3 d and 2 d later, respectively. Relative mRNA expression levels (measured by RT-qPCR) of *Pkm* or *GAPDH* were determined by normalization to expression levels of *Tbp*. Data represent mean of means +SEM (n = 3 for panels A and B, n = 4 for panels C and D). *, $p < 0.05$ vs. universal negative siRNA.

We hope that the reported protocol will be an easily accessible and useful tool for deciphering novel aspects of brown adipocyte biology and browning of white adipocytes *in vitro*.

Materials and methods

Culture of cell lines

The WT-1 cell line established by immortalization of brown pre-adipocytes from newborn mice with SV40 large T antigen was kindly provided by Dr. C. Ronald Kahn.[21] WT-1 cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, 52100) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, 10270), 62.5 μg/ml penicillin and 100 μg/ml streptomycin (Life Technologies, 15140–122). The 3T3-L1 white pre-adipocyte cell line[22] was propagated as WT-1 cells, except that 10% calf serum (PPA Laboratories, B15-004) was used instead of FBS. Two days post-confluent WT-1 and 3T3-L1 cells (designated day 0) were induced to differentiate in WT-1 propagation medium supplemented with 1 μM dexamethasone (Sigma-Aldrich, D1756), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, I5879), 5 μg/ml insulin (Roche, 11376497001) and 1 μM rosiglitazone (Cayman Chemicals, 71740). At day 2, cells were refreshed with medium containing 5 μg/ml insulin and 1 μM rosiglitazone. From day 4, the cells were cultured in WT-1 propagation medium.

hMADS cells[23] were obtained from Dr. Christian Dani and propagated in Advanced DMEM/F12 (Life Technologies, 12634) supplemented by 10% FBS, 2 mM

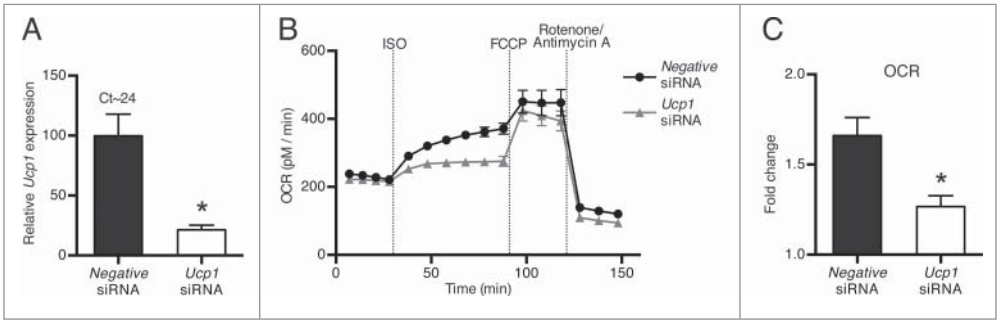

Figure 6. Functional consequences of *Ucp1* knockdown. Mature WT-1 brown adipocytes were reverse transfected at day 6 of differentiation with an siRNA against *Ucp1* or a universal negative siRNA. The cells were harvested 4 d later. (A) Relative mRNA expression levels (measured by RT-qPCR) of *Ucp1* were determined by normalization to expression levels of *Tbp*. (B) Oxygen consumption rate (OCR) was measured on a Seahorse XF96 Analyzer under basal conditions and during successive addition of 1 μM isoproterenol (ISO), 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and a mixture of 1 μM rotenone and 1 μM antimycin A. The figure shows a representative experiment (out of 4). (C) Fold change in OCR between the basal level and the measurement 1 h after ISO stimulation. Data represents mean of means +SEM (panels A and C) or mean ± SEM (panel B) (n = 4). *, $p < 0.05$ versus universal negative siRNA.

L-glutamine (Life Technologies, 25030), 62.5 μg/ml penicillin and 100 μg/ml streptomycin. Two days post-confluent cells (day 0) were induced to differentiate in Advanced DMEM/F12 with 2% FBS and 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 5 μg/ml insulin, 1 μM rosiglitazone, 1 μM cortisol (Sigma-Aldrich, H0135) and 1 nM thyroid hormone (T_3) (Sigma-Aldrich, T6397). This treatment was repeated at day 3. On day 6, the cells were refed with Advanced DMEM/F12 with 2% FBS containing 1 μM rosiglitazone and 1 nM T_3.

Cell cultures were kept at 37°C in a humidified atmosphere with 5% CO_2. Cells used for reverse siRNA transfections were cultured in 10 cm dishes until transfection.

Isolation and culture of primary adipocytes

Primary brown (from interscapular, cervical and axillary BAT) and inguinal white pre-adipocytes, from 3 to 4 weeks old NMRI mice (males and females), were isolated and cultured essentially as described.[24] After the tissue was minced and transferred to a HEPES-buffered solution (pH 7.4) containing 0.2% crude collagenase type II (Sigma-Aldrich, C6885), it was digested at 37°C for 30 min with constant shaking. The cell suspension was filtered through a 250-μm filter and incubated on ice for 15 min to separate the mature adipocytes and the stromal vascular (SV) fraction. The SV fraction was then filtered through a 50-μm filter. After centrifugation (10 min, 700 g), the pellet was resuspended in culture medium (DMEM, 4.5 g D-glucose/liter) (Sigma-Aldrich, D6429), 10% newborn calf serum (Life Technologies, 16010–159), 2.4 nM insulin (Novo Nordisk, 8–0204), 4 mM L-glutamine, 10 mM HEPES (Lonza, BE17-737E), 25 μg/ml sodium ascorbate (Sigma-Aldrich, A4034), 50 IU/ml penicillin and 50 μg/ml streptomycin and centrifuged. The 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 isolation.

Reverse siRNA transfections

Pre-adipocytes and mature adipocytes were reverse transfected with siRNA as follows: siRNA and Lipofectamine RNAiMAX (Life Technologies, 13778–150) were diluted in Opti-MEM I Reduced Serum Medium (Life Technologies, 31985-062) separately before being mixed by pipetting. The siRNA-RNAiMAX mix was added to gelatin-coated 96- or 48-well cell culture plates or 96-well Seahorse plates, and left to incubate for 25 min at room temperature. The final concentrations of Lipofectamine RNAiMAX and siRNA were 5 μl/ml and 50 nM, respectively, in 96-well cell culture and Seahorse plates, and 9 μl/ml and 90 nM, respectively, in 48-well plates. The final amount of medium per well was 90 μl in a Seahorse plates, 180 μl in 96-well culture plates and 250 μl in 48-well plates.

Pre-adipocytes were reverse transfected when the cells were ~70% confluent. Pre-adipocytes were detached with 0.05% trypsin (Sigma-Aldrich, T3924) for 5 min, counted and spun down (5 min, 300 g). The cells were resuspended in culture medium and added (20,000 cells per well) to 96-well plates on top of the pre-incubated siRNA-RNAiMAX mix. The cells were harvested 2 d after transfection.

Mature adipocytes were transfected at day 6 of differentiation for mouse cell lines (WT-1 and 3T3-L1), day 8 for primary cultures or day 9 for hMADS cells. Mature adipocytes were trypsinized with 0.25% trypsin (Life Technologies, 25200-072) for 15–20 min, counted and

spun down (5 min, 300 g). The cells were resuspended in culture medium and added to Seahorse, 96- or 48-well plates on top of the pre-incubated siRNA-RNAiMAX mix. The final number of cells per well was 20,000, 80,000 and 200,000 in Seahorse, 96- and 48-well culture plates, respectively. Reverse transfection and replating were performed in 96-well plates for mouse and human cell lines and in 48-well plates for primary cells. Figure 1 illustrates the protocol for reverse siRNA transfection.

Mature mouse primary adipocytes and hMADS cells were harvested 2 and 3 d after transfection, respectively. For the mouse cell lines (WT-1 and 3T3-L1 cells) the medium was changed after 2 d and the cells were harvested 4 d after transfection.

The MISSION® siRNAs (Sigma-Aldrich) used were mouse *Pkm* (SASI_Mm01_00036294), mouse *Camk2a* (SASI_Mm01_00347598) and mouse *Ucp1* (SASI_Mm01_00067600). For hMADS cells, the Silencer Select siRNA against human *GAPDH* (Life Technologies, 4427038) was used. The MISSION® siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001) was used as control in all mouse experiments and Negative Control #1 (Life Technologies, 4390843) was used as control in hMADS cells.

Forward siRNA transfections

Pre-adipocytes and mature adipocytes were forward transfected with siRNA when the cells were ~70% confluent or at day 6 of differentiation, respectively. As with the reverse siRNA transfection, the siRNA and Lipofectamine RNAiMAX were diluted separately and mixed by pipetting. The siRNA-RNAiMAX mix was left to incubate for 25 min at room temperature after which the siRNA-RNAiMAX mix was added on top of the adherent cells. The concentration of Lipofectamine RNAiMAX and siRNA was as described for the reverse siRNA transfection in 96-wells culture plates. The pre-adipocytes were harvested 2 d after transfection. For mature adipocytes the medium was changed after 2 d and the cells were harvested 4 d after transfection.

Oil red O staining

Oil red O staining was performed as previously described.[25]

Seahorse measurements

Four days after transfection, real-time measurements of OCR were performed using the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience). One h before the first measurement, the cell culture medium was changed to DMEM (Seahorse Bioscience, 102352) supplemented with 5 mM glucose (Sigma-Aldrich, G7021) and 2% bovine serum albumin (Sigma-Aldrich, A9647) (adjusted to pH 7.4). OCR was measured under basal conditions and during successive addition of 1 μM ISO (Sigma-Aldrich, I5627), 1 μM FCCP and a mixture of 1 μM rotenone and 1 μM antimycin A (Seahorse Bioscience, 101706–100).

RT-qPCR

Total RNA was purified using TRI Reagent (Sigma-Aldrich, T9424) according to the instructions of the manufacturer, and reverse transcription and qPCR were performed as previously described,[26] except that the SensiFAST SYBR Lo-ROX Kit was used (Bioline, BIO-94005). Primers used were: mouse *Camk2a*, fwd-TCTTC TGAGAGCACCAACAC, rev-GGTCGCACATCTTCGT GTA (136 bp); mouse *Fabp4*, fwd-TGGAAGCTTG TCTCCAGTGA, rev-AATCCCCATTTACGCTGATG (111 bp); mouse *Glut4*, fwd-ATCATCCGGAACCTG-GAGG, rev-CGGTCAGGCGCTTTAGACTC (52 bp); mouse *Pkm*, fwd-CTGTGGAGATGCTGAAGGAG, rev-CAACAGGACGGTAGAGAATGG (156 bp); mouse *Tbp*, fwd-ACCCTTCACCAATGACTCCTATG, rev-ATGATGACTGCAGCAAATCGC (190 bp); mouse *Ucp1*, fwd-AGCCGGCTTAATGACTGGAG, rev-TCTG TAGGCTGCCCAATGAAC (51 bp); human *GAPDH*, fwd-GCAAGAGCACAAGAGGAAGAG, rev-CTACAT GGCAACTGTGAGGAG (103 bp); human *TBP*, fwd-CCCGAAACGCCGAATATAA, rev-GAAAATCAGTG CCGTGGTTC (83 bp).

Whole cell extracts and immunoblotting

Cells transfected with *Pkm* siRNA were harvested 4 d after transfection. Preparation of whole-cell extracts and immunoblotting were done as described.[25] Antibodies used were against Ser473 phosphorylated AKT (Cell Signaling Technologies, 4058), GAPDH (Abcam, Ab8245) and PKM (Sigma-Aldrich, SAB4200095).

Glycerol release

Four days after transfection with control or *Pkm* siRNA, following addition of fresh medium, cells were stimulated with 1 μM ISO. Cell culture medium was collected after 6 h of stimulation and stored at −20°C. Glycerol release was measured using the Adipolysis Assay Kit (Cayman Chemical, 10009381) essentially following the instructions of the manufacturer.

Statistical analyses

For all cell culture studies, 3 samples were harvested for each condition in each experiment, except for primary cultures where only 2 wells were harvested. Data represent mean of means of 3 to 4 independent experiments + standard error of the mean (SEM). Statistical significance in RT-qPCR data and fold change in OCR was determined through 95% confidence intervals as calculated by an unpaired Student's t-test. Bonferroni correction was used when multiple comparisons were applied.

Abbreviations

BAT	brown adipose tissue
Camk2a	calcium/calmodulin-dependent protein kinase IIα
DMEM	Dulbecco's Modified Eagle's Medium
Fabp4	fatty acid-binding protein 4
FBS	fetal bovine serum
FCCP	carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
GAPDH	glyceraldehyde-3-phosphate
Glut4	glucose transporter 4
hMADS	human multipotent adipose-derived stem cells
ISO	isoproterenol
iWAT	inguinal white adipose tissue
OCR	oxygen consumption rate
Pkm	pyruvate kinase, muscle
RNAi	RNA interference
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SEM	standard error of the mean
shRNA	short-hairpin RNA
siRNA	small interfering RNA
SV	stromal vascular
T_3	thyroid hormone
Tbp	TATA-binding protein
Ucp1	uncoupling protein 1

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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