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Knockdown of *ZBED6* is not associated with changes in murine preadipocyte proliferation or differentiation

Wei Zhang[1], Mark A Cline[1], Dongmin Liu[2], and Elizabeth R Gilbert[1,*]

[1]Department of Animal and Poultry Sciences; Virginia Tech; Blacksburg, VA USA; [2]Department of Human Nutrition, Foods and Exercise; Virginia Tech; Blacksburg, VA USA

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ZBED6 was identified as a transcription factor that affects muscle mass and fat deposition in pigs. Mechanisms mediating effects on fat mass are unclear. The objective was to determine the effect of *ZBED6* mRNA knockdown on 3T3-L1 preadipocyte differentiation and gene expression. Differentiation was associated with increased mRNA abundance of *CEBP/α* ($P < 0.05$), *CEBP/β* ($P < 0.05$), *CEBP/δ* ($P < 0.05$), *FASN* ($P < 0.05$), *PPARγ* ($P < 0.05$), and *SREBP-1* ($P < 0.05$), and decreased abundance of *PREF-1* ($P < 0.05$). Knockdown of *ZBED6* was not associated with changes in mRNA abundance of selected genes, lipid accumulation, lipid droplet size, or cell number. These results suggest that ZBED6 does not play a major role in preadipocyte differentiation.

Introduction

Modern commercial pigs selected for lean meat production have increased skeletal muscle mass and reduced backfat thickness compared with their ancestor, the European wild boar. Gene mapping studies revealed a single nucleotide transition from G to A in intron 3 of the insulin-like growth factor 2 (*IGF2)* gene as being responsible for much of the difference in body composition between lean pigs and wild boars.[1] This mutation is located in a CpG site surrounded by a 16 bp evolutionarily conserved region.[1] It is associated with 3-fold greater postnatal expression of *IGF2* mRNA in skeletal muscle and heart and accounted for 3–4% increased skeletal muscle mass and reduced backfat thickness in pigs that carry the mutation on the paternal allele. The mechanism underlying the effect of the *IGF2* mutation is partly understood, as it disrupts binding with a recently identified transcription factor, ZBED6, a domesticated DNA transposon, unique to placental mammals, located in intron 1 of a "host" gene called *Zc3h11a*.[2,3] Chromatin immunoprecipitation experiments indicated that ZBED6 has thousands of potential target sites associated with growth, cell differentiation, transcriptional regulation, development, and neurogenesis in C2C12 mouse myoblast cells.[2] The role of ZBED6 in other tissues is unclear. Because the *IGF2* mutation is associated with reduced backfat accumulation in pigs, we hypothesized that ZBED6 might play a role in adipogenesis.

Adipocytes are derived from mesenchymal stem cells that differentiate into preadipocytes, when then terminally differentiate into adipocytes.[4] In vitro, following induction with differentiation media containing a cocktail of insulin, dexamethasone, and isobutylmethylxanthine (a non-selective phosphodiesterase inhibitor), preadipocytes will undergo growth arrest and one round of clonal expansion, followed by terminal differentiation. Adipose tissue accumulation involves extensive cellular remodeling and is dependent on the coordinated interplay between adipocyte hypertrophy and hyperplasia. There is no evidence that ZBED6 and IGF2 are directly associated with fat deposition or that ZBED6 regulates *IGF2* transcription in adipocytes. In this study we investigated the effect of *ZBED6* knockdown on adipocyte differentiation and *IGF2* expression with the goal to provide novel insight on the function of ZBED6 as well as molecular mechanisms underlying adipocyte differentiation and fat deposition.

Results and Discussion

Because the *IGF2* mutation discovered in pigs is associated with enhanced muscle mass and reduced backfat, with the mechanism involving the release of postnatal ZBED6-mediated transcriptional repression of *IGF2* in skeletal muscle, we hypothesized that ZBED6 may also play a role in regulating adipose tissue expansion. In this study we evaluated the effects of knocking down *ZBED6* mRNA on 3T3-L1 mouse preadipocyte proliferation and differentiation. There was 81% knockdown efficiency at 48 h post-*ZBED6* siRNA transfection. Knockdown efficiency was reduced from 81% at 48 h to 40% at 96 h post-transfection. It is possible that ZBED6 exerts effects at later stages of differentiation; however with the transient nature of siRNA transfections is difficult to assess the role of ZBED6 at later stages of differentiation without a stable knockdown approach. Cell

*Correspondence to: Elizabeth R Gilbert; Email: egilbert@vt.edu
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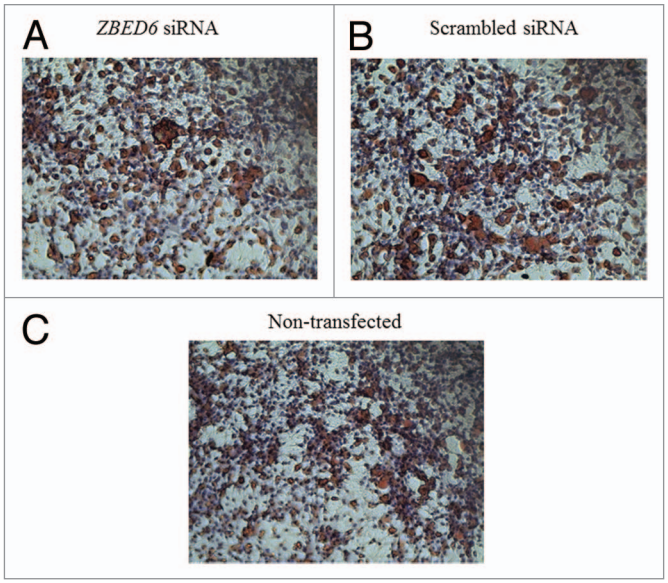

Figure 1. Oil Red O staining at day 7 post-induction of differentiation in zinc finger, BED-type containing 6 (*ZBED6*) siRNA-transfected (**A**), scrambled siRNA (**B**), and non-transfected 3T3-L1 cells (**C**). Differentiation was induced at post-transfection. $n = 3$ experiments.

viability was evaluated to investigate how *ZBED6* knockdown affects preadipocyte proliferation, as knockdown in C2C12 mouse myoblasts was associated with increased proliferation at 3 d post-silencing.[2] There were no significant differences at 48 or 216 h post-siRNA transfection (day 0 and 7 relative to initiation of differentiation, respectively) in cell viability (normalized relative absorbance) between *ZBED6* siRNA-transfected cells and scrambled siRNA cells (overall treatment means: 1.04 ± 0.016 vs. 1.05 ± 0.016, respectively; $P = 0.6$). Similarly, there were no changes in lipid accumulation between the *ZBED6* siRNA group (0.081 ± 0.002) and scrambled siRNA cells (0.084 ± 0.002) at 7 d post-differentiation ($P = 0.5$), as measured by absorbance following Oil Red O staining (**Fig. 1**). The area and numbers of adipocytes were also not different among treatment groups.

The mRNA abundance results for non-transfected cells showed that several genes were up- or downregulated during 3T3-L1 cell differentiation (**Table 1**). Differentiation was associated with an increase in *CEBP*/α (>15-fold; $P < 0.05$), *CEBP*/β (2-fold; $P < 0.05$), *CEBP*/δ (>20-fold; $P < 0.05$), *FASN* (1.5-fold; $P < 0.05$), *PPAR*γ (>15-fold; $P < 0.05$), and *SREBP-1* (5-fold; $P < 0.05$) mRNA at days 4 and 7 relative to day 0, and decrease in *PREF-1* (2-fold; $P < 0.05$) at 4 d post-induction of differentiation compared with day 0 (**Table 1**). These results are consistent with other reports of transcriptional events mediated during adipocyte differentiation.[5]

Adipocyte differentiation was not associated with a change in *ZBED6* or *IGF2* mRNA abundance, although it should be noted that both preadipocytes and adipocytes expressed very low levels of *IGF2* as estimated from raw C_T data, which may also explain the high variability among the biological replicates. Knockdown of *ZBED6* was not associated with any differences in expression of adipogenesis-associated factors or *IGF2* (**Table 2**). These results are in contrast to effects in myoblasts, where knockdown of *ZBED6* was associated with enhanced differentiation into myotubes at 6 d post-induction of differentiation.[2] Insulin-like growth factor 2 plays a role in tumor progression, and there was a report that increased expression of *IGF2* was associated with reduced adipocyte differentiation in human hemangioma cells.[6] In the present study, mRNA abundance of *ZBED6* and *IGF2* was measured in skeletal muscle and white adipose tissue of adult mice, with approximately 2-fold greater expression of both genes in skeletal muscle (**Fig. 2**), consistent with the idea that ZBED6 plays a more prominent role in skeletal muscle as compared with fat.

In summary, we did not find any evidence that ZBED6 plays a role in mouse preadipocyte proliferation or differentiation, or that *IGF2* expression in these cells was dependent on the presence of ZBED6. It is possible that *IGF2* does not play a critical role in the maintenance and differentiation of 3T3-L1 cells, as expression was almost undetectable and was not affected by induction of differentiation. These results are consistent with the current theory that the effect of the *IGF2* mutation in pigs is to partition energy toward muscle mass accretion at the expense of white adipose tissue accumulation (i.e., dependent on *IGF2* expression in skeletal muscle rather than adipose tissue).[2,3] One caveat to the present study though, is that 3T3-L1 cells are a clonal mouse-derived cell live with an unstable karyotype and may not be representative of in vivo adipogenesis between fat depots of different species. Expression and secretion of IGF2 was reported in white adipose tissue of humans[7] and neonatal pigs,[8] and in pigs it was demonstrated that *IGF2* was expressed in the stromal-vascular fraction of white adipose tissue with expression decreasing after induction of adipocyte differentiation.[9] Our real-time PCR results are consistent with a report showing by northern blot, a far less sensitive method for detecting mRNA, that *IGF2* was not expressed in 3T3-L1 cells.[10] With respect to transcriptional regulation of *IGF2* in 3T3-L1 cells, it is possible that ZBED6 doesn't repress *IGF2* in these cells or that other co-factors also contribute to suppressing *IGF2* expression in 3T3-L1 cells. Moreover, ZBED6 may play a different role in fatness, through effects on adipocyte hypertrophy, which was not addressed in this study because of the multilocular nature of differentiated 3T3-L1 cells.

Materials and Methods

Animals. All procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Six month-old male C57B6/N mice were obtained from the National Cancer Institute of NIH and housed individually in standard sized cages (29 × 14 × 13 cm) arranged in a double-faced 140-cage ventilation rack in a temperature and humidity controlled, pathogen-free room on a 12 h light cycle (6 AM to 6 PM) with free access to a standard rodent chow (Research Diet, Inc.). Eight animals were euthanized by CO_2 asphyxiation after an overnight fast and gonadal fat pads and gastrocnemius skeletal muscle removed and submerged in RNAlater (Qiagen).

Table 1. Gene expression in non-transfected 3T3-L1 cells at 0, 4, and 7 d post-induction of adipocyte differentiation*

Day	*C/EBP*α	*C/EBP*β	*C/EBP*δ	*FASN*	*GPDH*	*IGF2*	*PPAR*γ	*PREF-1*	*SREBP-1*	*ZBED6*
0	1.0 ± 5.7[b]	1.0 ± 0.1[b]	1.1 ± 2.7[b]	1.0 ± 0.1[b]	1.08 ± 0.4[a]	1.7 ± 16[a]	1.02 ± 2.8[b]	1.00 ± 0.1[a]	1.00 ± 0.3[c]	1.04 ± 0.2[a]
4	17.8 ± 5.7[a]	2.1 ± 0.1[a]	21.9 ± 2.7[a]	1.6 ± 0.1[a]	1.04 ± 0.4[a]	28.8 ± 16[a]	15.84 ± 2.8[a]	0.59 ± 0.1[b]	5.22 ± 0.3[a]	2.04 ± 0.2[a]
7	16.1 ± 7.0[a]	1.7 ± 0.1[a]	16.9 ± 3.3[a]	1.3 ± 0.1[a]	1.4 ± 0.5[a]	13 ± 19[a]	11.54 ± 3.5[a]	1.01 ± 0.1[a]	2.5 ± 0.3[b]	1.75 ± 0.3[a]

*LSmeans ± pooled SEM; mRNA abundance calibrated to day 0 within a gene. Different letter within a column indicates $P < 0.05$, Tukey test, $n = 3$ experiments.

Table 2. Effects of *ZBED6* mRNA knockdown on gene expression at 0, 4, and 7 d post-differentiation*

Treatment	Day	*C/EBP*α	*C/EBP*β	*C/EBP*δ	*FASN*	*GPDH*	*PPAR*γ	*PREF-1*	*SREBP-1*	*IGF2*	*ZBED6*
Scrambled	0	1.0 ± 0.7	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 5.4	1.07 ± 0.2[a]
siZBED6	0	0.7 ± 0.7	0.7 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	0.6 ± 0.3	0.8 ± 0.3	0.9 ± 0.1	1.1 ± 0.1	1.7 ± 5.4	0.2 ± 0.2[b]
Scrambled	4	1.3 ± 0.7	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	4.4 ± 5.4	1.1 ± 0.2[a]
siZBED6	4	1.9 ± 0.7	1.0 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.3	1.3 ± 0.3	1.0 ± 0.1	1.1 ± 0.1	7.6 ± 5.4	0.4 ± 0.2[b]
Scrambled	7	1.4 ± 0.8	1.0 ± 0.1	1.2 ± 0.3	1.0 ± 0.1	1.3 ± 0.4	1.3 ± 0.4	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 6.6	1.09 ± 0.2
siZBED6	7	1.8 ± 0.8	1.0 ± 0.1	0.9 ± 0.3	0.9 ± 0.1	1.2 ± 0.4	1.2 ± 0.4	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 6.6	0.8 ± 0.2

*LSmeans ± pooled SEM; mRNA abundance calibrated to the scrambled siRNA group within a time point. Different letter within a column and day indicates $P < 0.05$, Tukey test, $n = 3$ experiments.

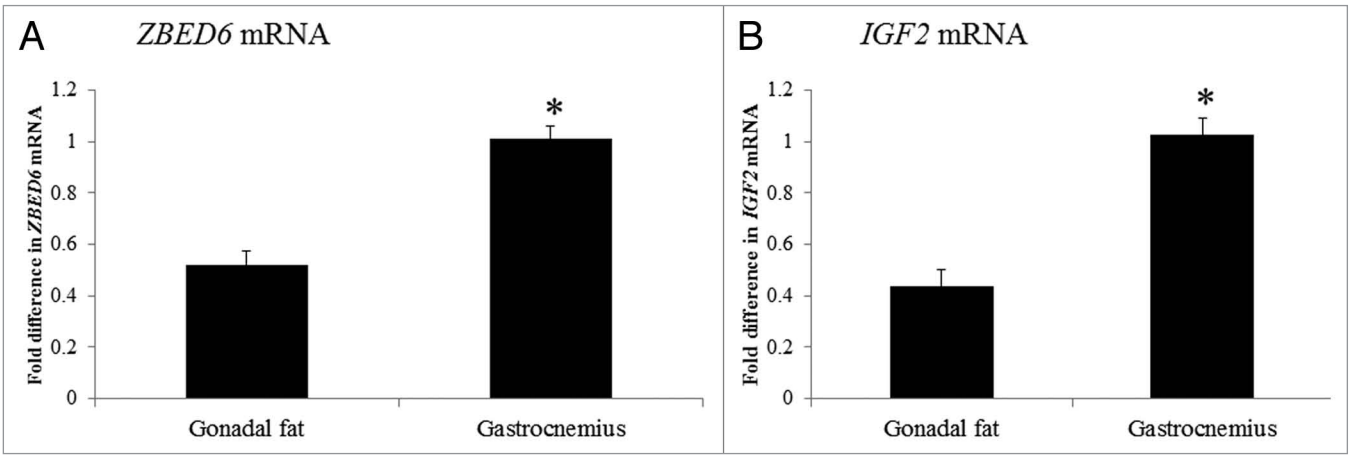

Figure 2. Relative abundance of zinc finger, BED-type containing 6 (*ZBED6*) (**A**) and insulin-like growth factor 2 (*IGF2*) (**B**) mRNA in gonadal fat and gastrocnemius skeletal muscle of 6-mo-old male C57B6/N mice. Values represent LSmeans ± pooled SEM ($n = 8$). *$P < 0.0001$.

Cell culture. The 3T3-L1 cells (Eton Bioscience Inc.) were cultured in preadipocyte growth media (DMEM high-glucose, 10% fetal bovine serum, penicillin/streptomycin; Hyclone, Thermo Fisher Scientific) at 37 °C in a humidified atmosphere of 5% CO_2, according to the supplier's instructions. Culture medium was changed every other day. At 70–80% confluence, cells were trypsin-digested for further sub-culturing or seeded into 12-well plates (Falcon) for siRNA transfection and induction of differentiation. Passages less than 4 were used for these experiments.

RNA interference. Three *ZBED6* specific siRNA oligonucleotides were designed to target three different regions of mouse *ZBED6* mRNA (Ambion).[2] Scrambled siRNA (silencer select No. 2; Ambion) was used as a negative control.[2] The RNA interference was performed as previously described.[2] Briefly, suspensions containing 100 000 cells/ml were reverse transfected in triplicate with siRNA using lipofectamine 2000 (Invitrogen) and Optimem-I reduced serum media (Invitrogen) with the 3 siRNAs pooled at equal amounts to a final concentration of 50 nM. Triplicate wells of non-transfected cells were also included. To evaluate the effects of *ZBED6* knockdown on cell growth and morphology, cells were imaged daily with a digital inverted microscope (EVOS). The average of the triplicates within an assay was considered the experimental unit and the experiment was repeated at least 3 times on independent passages of cells.

Adipocyte differentiation. Preadipocytes were induced to differentiate into adipocytes based on the following induction protocol for 2 weeks: At 48 h post-siRNA transfection, media was changed to complete adipogenic induction media (growth media containing 5 μg/ml insulin (Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine; Sigma) and incubated for 3 d, and then changed to maintenance media

Table 3. Primers used for real time PCR

Gene*	Accession number	Sequences (forward/reverse)
PREF-1	NM_001190705.1	CCAACGTGAC CAAAGATCAA GA
		GGATGCCGTG GAATTTTGAC
C/EBPα	NM_007678.3	CAGTTGGGCA CTGGGTGGGC
		CCGCGGCTCC ACCTCGTAGA AG
C/EBPβ	NM_009883.3	CGCAACACAC GTGTAACTGT CA
		AACAACCCCG CAGGAACAT
C/EBPδ	NM_007679.4	TCCAACCCCT TCCCTGATC
		CCCTGGAGGG TTTGTGTTTT C
SREBP-1	NM_011480.3	GCCTAGTCCG AAGCCGGGTG
		GGAGCATGTC TTCGATGTCG TTCA
PPARγ	NM_001127330.1	GCCTGCGGAA GCCCTTTGGT
		AAGCCTGGGC GGTCTCCACT
FASN	NM_001146708.1	TGCCAACCTG AAAACTAGGC TGAG
		TACCCACCCC ACCCCCTTCT C
GPDH	NM_001145820.1	AGAGCTGCAG GCCGAGTCCC
		GCTCAGCCTG ATCACCCGTC GC
ZBED6	NM_001166552.1	CAAGACATCT GCAGTTTGGA ATTT
		TGTCGTTGAA GTGTTGAAGT TCCTA
IGF2	NM_001122737.1	CGTGGCATCG TGGAAGAGT
		ACACGTCCCTCT CGGACTTG
18S	NR_003278.3	ACCTGGTTGA TCCTGCCAGT AG
		TTAATGAGCC ATTCGCAGTT TC

*Primers were designed for a variety of genes associated with adipogenesis, as reviewed in reference 12. Preadipocyte Factor 1 (*PREF-1*) encodes a preadipocyte secreted factor that serves as a marker for preadipocytes. The CCAAT/enhancer binding protein (C/EBP) α and β activate expression of PPARγ and are required for preadipocyte differentiation, while C/EBP/δ and sterol regulatory element-binding protein-1 (SREBP-1) accelerate but are not required for differentiation. Peroxisome proliferator-activated receptor γ (PPARγ) is the master transcriptional regulator of adipogenesis and is involved in the growth arrest that is required for differentiation. Also investigated in this study was expression of fatty acid synthase (*FASN*), a key enzyme in de novo lipogenesis that catalyzes the synthesis of saturated fatty acids, and glycerol-3-phosphate-dehydrogenase (*GPDH*), an enzyme that catalyzes the reversible conversion of dihydroxyacetone phosphate to sn-glycerol-3-phosphate. Expression of zinc finger, BED-type containing 6 (*ZBED6*) and insulin-like growth factor 2 (*IGF2*) was also evaluated. The 18S ribosomal subunit served as the endogenous control.

(growth media containing 5 μg/ml insulin, 1 μM dexamethasone). Maintenance media was replaced every two days.

Cell viability assay. Cell viability was evaluated at day 0 (48 h post-siRNA transfection), and days 1 and 8 post-induction of differentiation with Alamar blue reagent (Invitrogen). Alamar blue reagent was added to each well of 12-well plates and incubated for 1 to 4 h. Absorbance was measured at 570 nm (reduced) and 600 nm (oxidized) using a multi-mode plate reader (M200 Pro; Tecan Instruments). Cell viability was calculated as the ratio of reduced/oxidized relative to the negative control group.

Oil Red O staining. Cells were fixed with 10% formalin for 30 min at room temperature and Oil Red O staining performed according to the manufacturer's instructions (American Master Tech). Propylene glycol was added to each well and incubated for 5 min, replaced with Oil Red O working solution and incubated for another 5 min at room temperature, and rinsed with water. Absorbance was measured at 490 nm to estimate lipid accumulation. Cells were then counterstained with hematoxylin and imaged to estimate the number of adipocytes and size of lipid droplets.

Total RNA isolation and real time PCR. At 48, 144, and 216 h post-siRNA transfection (Day 0, 4, and 7 relative to induction of adipocyte differentiation), cells were washed with phosphate-buffered saline and lysed with a 21 gauge needle in 350 μL RLT buffer (Qiagen). The total RNA was extracted with the RNeasy Mini kit (Qiagen Sciences) according to the manufacturer's instructions. An on-column RNase-Free DNase I (Qiagen) treatment was incorporated to eliminate genomic DNA carry-over in the RNA preparations. The eluted total RNA samples were quantified and assessed for purity by spectrophotometry at 260/280/230 nm using a Nanophotometer™ Pearl (IMPLEN), and integrity evaluated by agarose gel electrophoresis. The first strand cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primers were designed in Primer Express 3.0 (Applied Biosystems; **Table 3**). All primers were evaluated for amplification efficiency before use. Efficiency of target genes was within 5% of the house keeping gene (18S rRNA). A total volume of 10 μl in each reaction contained 5 μl fast SYBR Green Master Mix (Applied Biosystems), 0.25 μl each of 5 μM forward and reverse primers, and 1 μl of cDNA. Real-time PCR reactions were performed in duplicate for all samples on an Applied Biosystems 7500 FAST system, under the following conditions: enzyme activation for 20 s at 95 °C and 40 cycles of 1) melting step for 3 s at 95 °C and 2) annealing/extension step for 30 s at 60 °C. Melting curve analysis was performed after all reactions to ensure amplification specificity.

For tissue samples collected in RNAlater, approximately 200 mg was homogenized with a Tissue Lyser II (Qiagen) twice at 20 Hz for 2 min using 5 mm stainless steel beads (Qiagen) and 1 ml isol-RNA lysis reagent (5-PRIME). Samples were then centrifuged at 12000 × g for 10 min at 4 °C, supernatant transferred and mixed with 0.2 ml chloroform, and centrifuged again under the same conditions. The supernatant was removed and mixed with an equal volume of 70% ethanol and loaded onto a spin column and total RNA purified according to the manufacturer's instructions (Qiagen RNeasy Mini). An on-column RNase-Free DNase I (Qiagen) treatment step was included. Reverse transcription and real time PCR were performed as described above.

Statistical analysis. The real time PCR data for cells were analyzed using the $\Delta\Delta C_T$ method, where $\Delta C_T = C_{T\ \text{target gene}} - C_{T\ 18S}$, and $\Delta\Delta C_T = \Delta C_{T\ \text{target sample}} - \Delta C_{T\ \text{calibrator}}$.[11] To evaluate the effect of *ZBED6* knockdown on gene expression, the negative control within a time point was used as the calibrator sample. In non-transfected cells, the day 0 values were used as the calibrator to evaluate changes in gene expression during differentiation. The relative quantity ($2^{-\Delta\Delta Ct}$) values were subjected to ANOVA using the Proc Glimmix procedure of SAS. The statistical model

included the main effect of treatment for *ZBED6*-knockdown cells and main effect of time for non-transfected cells. A similar model was used for cell viability (normalized absorbance), lipid accumulation (Oil Red O normalized absorbance), and cell morphology data. The Tukey test was used for pairwise comparisons across time points. For tissue real-time PCR data, the skeletal muscle was used as the calibrator, and differences between muscle and fat tested using the Student *t* test. Results were considered significant at $P < 0.05$.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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