TWIST1 and TWIST2 regulate glycogen storage and inflammatory genes in skeletal muscle

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Abstract

TWIST proteins are important for development of embryonic skeletal muscle and play a role in the metabolism of tumor and white adipose tissue. The impact of TWIST on metabolism in skeletal muscle is incompletely studied. Our aim was to assess the impact of TWIST1 and TWIST2 overexpression on glucose and lipid metabolism. In intact mouse muscle, overexpression of Twist reduced total glycogen content without altering glucose uptake. Expression of TWIST1 or TWIST2 reduced Pdk4 mRNA, while increasing mRNA levels of Il6, Tnfα, and Il1β. Phosphorylation of AKT was increased and protein abundance of acetyl CoA carboxylase (ACC) was decreased in skeletal muscle overexpressing TWIST1 or TWIST2. Glycogen synthesis and fatty acid oxidation remained stable in C2C12 cells overexpressing TWIST1 or TWIST2. Finally, skeletal muscle mRNA levels remain unaltered in ob/ob mice, type 2 diabetic patients, or in healthy subjects before and after 3 months of exercise training. Collectively, our results indicate that TWIST1 and TWIST2 are expressed in skeletal muscle. Overexpression of these proteins impacts proteins in metabolic pathways and mRNA level of cytokines. However, skeletal muscle levels of TWIST transcripts are unaltered in metabolic diseases.

Key Words

TWIST
metabolism
glycogen
skeletal muscle

Introduction

The transcription factors TWIST1 and TWIST2 (also known as DERMO1) are genes belonging to the basic helix–loop–helix (bHLH) transcription factor family, originally described in Drosophila and conserved in humans (Thiss et al. 1987). TWIST1 and TWIST2 form homo- or heterodimers, which together with other bHLH family members bind to the E-box DNA sequence 5'-NCANNTGN-3' (Castanon et al. 2001). The resulting transcriptional activity depends on post-transcriptional modifications, partner choice, and cellular context, thus complicating the characterization of modes of action of the TWIST proteins (Laursen et al. 2007).

TWIST1 and TWIST2 have overlapping but not identical effects, with TWIST1 being the more thoroughly investigated gene. TWIST proteins play important roles in tissue differentiation. In skeletal muscle, TWIST1 blocks myogenesis via inhibition of MYOD transactivation (Hamamori et al. 1997), which may lead to myotube dedifferentiation (Hjiantoniou et al. 2008). TWIST proteins have also been implicated in cancer as they block p53 and Myc-dependent apoptosis (Maestro et al. 1999). Moreover TWIST1 facilitates the appearance of metastasis by promoting cell migration (Yang et al. 2004), and its expression level correlates with metastasis and poor
prognosis in cancer (Hosono et al. 2007, Ou et al. 2008). Mutations in TWIST1 are responsible for Saethre–Chotzen syndrome (OMIM 101400) characterized by craniosynostosis and limb abnormalities. In mice, homozygous deletion of the Twist1 gene results in embryonic lethality, while mice with a deletion of the Twist2 gene die 3 days after birth due to cachexia with high levels of pro-inflammatory cytokines and a complete absence of glycogen stores (Sosic et al. 2003).

A role for TWIST proteins in metabolism has been proposed in adipose tissue, where TWIST1 is reported to regulate cytokine expression (Pettersson et al. 2010). In adipose cells, TWIST1 silencing reduces fatty acid oxidation and modulates pro-inflammatory cytokine expression, in particular IL6 (Dobrian 2012). Furthermore, reduced TWIST1 expression in human white adipose tissue has been correlated with insulin-resistance and obesity (Pettersson et al. 2011). TWIST1 is also a negative feed-back regulator of PGC1α/PPARγ-mediated signaling in brown fat (Pan et al. 2009). The role of TWIST proteins in mature skeletal muscle is less well explored. Based on reported effects of TWIST1 in adipose cells, we hypothesized that TWIST proteins may be involved in skeletal muscle glucose and lipid metabolism, as well as in the regulation of expression of skeletal muscle-derived cytokines.

Materials and methods

Human subjects

Male and female volunteers with type 2 diabetes mellitus (T2D) or normal glucose tolerance (NGT) were matched for weight and BMI. Detailed clinical characteristics of the patients are given in Table 1. As expected, fasting and 2 h glucose levels post oral glucose tolerance test were increased in T2D patients. Patients on insulin treatment and with symptomatic coronary heart disease were excluded. A muscle biopsy of the vastus lateralis and anthropomorphic measurements were taken at enrolment in the study (Barres et al. 2012, Kulkarni et al. 2012). In a separate cohort, 13 sedentary volunteers participated in an exercise training program consisting of 60 min a day, 5 days/week for 3 weeks. Biopsies of the vastus lateralis muscle were obtained before the first and 48 h after the last training bout. This exercise training program increased VO2 max by 13% (P<0.05; Czepluch et al. 2011). The clinical parameters and detailed of the exercise protocol are reported elsewhere (Czepluch et al. 2011). All studies were performed according to the declaration of Helsinki, with informed consent and approved by the ethics committee at Karolinska Institute.

Table 1 Anthropometric measurements and metabolic parameters of human volunteers. Data are presented as means ± S.E.M.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NGT</th>
<th>T2D</th>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>5/5</td>
<td>4/6</td>
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<tr>
<td>Age (y)</td>
<td>58 ± 2</td>
<td>63 ± 1*</td>
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<tr>
<td>Height (cm)</td>
<td>171 ± 3</td>
<td>170 ± 4</td>
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<td>Weight (kg)</td>
<td>84.8 ± 3.6</td>
<td>90.8 ± 5.8</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>93.2 ± 3</td>
<td>106.0 ± 4*</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>29.1 ± 0.6</td>
<td>31.3 ± 1.2</td>
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<tr>
<td>SBP (mmHg)</td>
<td>133 ± 5</td>
<td>140 ± 3</td>
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<tr>
<td>DBP (mmHg)</td>
<td>79 ± 3</td>
<td>78 ± 2</td>
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<td>FBG (mmol/l)</td>
<td>5.5 ± 0.0</td>
<td>7.9 ± 0.2**</td>
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<tr>
<td>2-h BG (mmol/l)</td>
<td>6.6 ± 0.4</td>
<td>14.5 ± 1.4*</td>
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<tr>
<td>HbA1c (%)</td>
<td>4.6 ± 0.1</td>
<td>5.3 ± 0.2*</td>
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<tr>
<td>Insulin (pmol/l)</td>
<td>51.4 ± 12.5</td>
<td>92.5 ± 16.6*</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.45 ± 0.33</td>
<td>1.24 ± 0.05*</td>
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<tr>
<td>LDL (mmol/l)</td>
<td>3.24 ± 0.18</td>
<td>2.74 ± 0.28</td>
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<tr>
<td>TG (mmol/l)</td>
<td>51.4 ± 12.8</td>
<td>92.5 ± 16.9</td>
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<tr>
<td>IL6 (pg/ml)</td>
<td>1.54 ± 0.37</td>
<td>2.23 ± 0.51</td>
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<tr>
<td>HOMA-IR</td>
<td>1.97 ± 0.10</td>
<td>3.48 ± 0.30**</td>
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NGT, normal-glucose tolerant; T2D, type 2 diabetes; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; TG, triglycerides; HOMA, homeostatic model assessment of insulin resistance. **P<0.01; *P<0.05.

Mice

C57BL/6J mice (12–14 weeks old) were purchased from Charles River (Sulzfeld, Germany) and acclimatized for at least 1 week before use. Mice were housed in a humidity and temperature-controlled environment with 12 h light:12 h darkness cycle and provided ad libitum access to water and standard rodent chow (4% fat, 16.5% protein, 58% carbohydrates, 3.0 kcal/g purchased from Lantmännen, Stockholm, Sweden). Tissue collection from C57BL/6J- ob/ob and lean +/+ female mice at 14–16 weeks of age was performed after anesthesia using 0.02 ml/g body weight of Avertin (2.5% solution of 99% 2,2,2-tribromo ethanol and tertiary amyl alcohol purchased from Sigma–Aldrich). The muscles were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. The study protocols were approved by the animal ethics committee of Stockholm north.

Plasmid construct

pZsGreen1-C1 construct was purchased from Clontech. The pZsGreen1 (GFP) sequence was removed and replaced with Twist1 or Twist2 sequences. The inserted sequences were species-optimized for mice by Geneart (Regensburg, Germany). The Twist1 and Twist2 DNA sequences were created by GeneArt using the mouse protein sequence for TWIST1 (Swiss-Prot: P26687.1) and TWIST2 (Swiss-Prot:
Gene transfer by electroporation in intact skeletal muscle and metabolic analysis

Male C57BL/6 mice (12–14 weeks old) were purchased from Charles River and acclimatized for at least 1 week before use. Mice were housed in a humidity- and temperature-controlled environment with 12 h light:12 h darkness cycle and provided ad libitum access to water and standard rodent chow. Tibialis anterior muscles of adult C57BL/6J mice were transfected with either an empty vector or the vector encoding for TWIST1 or TWIST2 (Clontech) by electroporation as described previously (Kulkarni et al. 2011).

One week after electroporation, mice were fasted for 4 h and subjected to a modified oral glucose tolerance test to assess glucose uptake into skeletal muscle, as described and subjected to a modified oral glucose tolerance test to assess glucose uptake into skeletal muscle, as described

mRNA extraction and analysis

mRNA was extracted from 10 mg of skeletal muscle tissue homogenized in 1 ml of TRIzol reagent (Invitrogen) and was purified according to the manufacturer’s recommendations. For mRNA extraction from cultured cells, myoblasts were washed three times with PBS and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and purified with DNase (Qiagen) according to recommendations of the manufacturer.

RNA concentration was measured with Nanodrop 1000 (Thermo Scientific) and reverse transcribed using the High-capacity cDNA RT Kit (Applied Biosystems). Real-time PCRs were performed in duplicate using TaqMan-based probes in a Step One Plus detector (Applied Biosystems). GAPDH (Hs00446968_m1 for human samples and Mm99999905_m1 for mouse samples) was used as an endogenous control. For mRNA measurement after electroporation, results were compared against the geometrical mean of three different housekeeping genes: GAPDH, HPRT1 (Mm00446968_m1), and TBP (Mm00446971_m1). The following TaqMan primer and probe sets from Applied Biosystems were used: for mouse origin: Il1b: Mm00434228_m1, Tnfa: Mm00443260_g1, Il6: Mm00446190_m1, myogenin: Mm00446195_g1, MyoD: Mm01203489_g1, Pax7: Mm01354484_m1, Twist1: Mm00442036_m1, Twist2: Mm00492147_m1 and for human origin: Twist1: Hs01675818_s1, Twist2: Hs02379973_s1. For measurement of plasmid product, custom-made TaqMan primers were designed (Sigma-Aldrich) with the following sequence: Twist1: forward: GAACCTGCCAGCCAGCGGT, probe: CCTGAACGGGCTTGCAGC, and reverse: CCGTGAGGGCCTTCGGCG; Twist2: forward: ACGGCCAGACGCTGGAGGA, probe: GCCAAGCTGCGAGAGACA, and reverse: CGGCGAAGGCTTCAGG.

C2C12 mouse cell line and plasmid transfection

C2C12 cells were obtained from ATCC (Manassas, VA, USA). The cells were grown in high-glucose DMEM (Gibco) with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% penicillin–streptomycin (Gibco), and 1% fungizone (Gibco). The medium was changed every second day. C2C12 myoblasts (between passages 9 and 12) were seeded at 2×10⁴ cells/cm² in a six-well plate (Corning, New York, NY, USA) for 24 h and then transfected with 2 μg Twist1 or Twist2 DNA construct (1 μg/μl) and 6 μl/ml of FuGENE HD (Promega) or FuGENE HD only (as control) in DMEM containing 10% FBS but without antibiotic or antifungal additions for 24 h. Each condition was performed in triplicate.

Cytokine measurement in C2C12 myoblasts-conditioned media

Following 24 h in transfection medium, C2C12 myoblasts were incubated in a serum-free medium. After 2 h, the
medium was changed to a fresh serum-free medium and cells were incubated for 16 h. The medium was then collected and IL6, IL1β, and the TNFα contents in the conditioned culture media were assessed by ELISA following the manufacturer’s instructions (Life Technologies; catalogue items: KMC0061, KMC0011, and KMC3011).

**Immunoblot analysis**

Skeletal muscle lysate was diluted in Laemmli buffer solution and separated by SDS–PAGE. After transfer to nitrocellulose membranes (Bio-Rad), proteins were blocked in 7.5% nonfat milk, washed with TBST (10 mmol/l Tris–HCl, 100 mmol/l NaCl, and 0.02% Tween 20), and incubated with primary antibody overnight at 4°C. The membranes were washed with TBST and incubated with appropriate HRP-conjugated secondary antibodies (Bio-Rad, diluted 1:25 000). Proteins were visualized by ECL (GE Healthcare, Little Chalfont, UK) and quantified using Quantity One Software (Bio-Rad). Primary antibody for glycogen synthase (GS) (catalogue item: #3893), GS phosphorylated on serine 641 (p-GS) (#3891), ACC (T#3676), ACC phosphorylated on serine 79 (p-ACC) (#3661), AKT (or protein kinase B (PKB)) (#9272), and its phosphorylated form on serine 473 (p-AKT) (#9271) were purchased from Cell Signaling (Danvers, MA, USA), GAPDH (#sc-2577) was purchased from Santa-Cruz Biotechnology and TWIST2 (#ab66031) from Abcam (Cambridge, UK). Additional TWIST1 antibodies from Santa-Cruz Biotechnology and TWIST1(#9271) were purchased from Santa-Cruz Biotechnology and TWIST2(#ab66031) from Abcam (Cambridge, UK). Additional TWIST1 antibodies from Santa-Cruz Biotechnology (#sc-15393 and #sc-6269) and Sigma–Aldrich (#T6451) were also used.

**Glycogen synthesis in C2C12 myoblasts**

Glucose incorporation to glycogen was determined in C2C12 myoblasts as previously described (Al-Khalili et al. 2003). In brief, after 24 h in transfection medium, cells were serum starved for 2 h. The myoblasts were treated with 120 nM insulin for 30 min before adding 1 mCi/mI D-[U-14C] glucose (1 mCi/ml and 289 mCi/mmol; Perkin Elmer, Waltham, MA, USA) for the last 90 min. Following incubation, monolayers were washed with ice-cold PBS and lysed in 0.5 ml 0.03% SDS. The suspension (0.4 ml) was transferred to 2-ml tubes and 0.1 ml (2 mg/sample) carrier glycogen was added. The remaining cell suspension was used for protein concentration determination by the Pierce BCA Protein Assay Kit (Thermo Scientific). The samples were heated to 99°C for 1 h. Glycogen was precipitated by addition of 95% ethanol and incubated overnight at −20°C. Glycogen pellets were collected by centrifugation for 15 min at 11 000 g, washed once with 70% ethanol, and resuspended in 0.3 ml distilled water. [14C]labeled glycogen was counted in a liquid scintillation counter (WinSpectral 1414, Wallac). Each experiment was performed in triplicate. Data are average of four independent experiments.

**Palmitate oxidation in C2C12 myoblasts**

Palmitate oxidation was determined in triplicate samples as previously described (Rune et al. 2009), including nonradioactive palmitate. In brief, 24 h after transfection, C2C12 myoblasts were serum starved for 2 h in DMEM. The medium was changed to 1 ml DMEM containing 25 μM of palmitate including 0.078 μM [3H][9,10-3H(N)] palmitate (5 mCi/ml and 32.0 Ci/mmol) (Perkin Elmer) and 0.04% BSA. The medium was collected 6 h later and cells were harvested for protein determination. In brief, 0.2 ml of collected medium was incubated for 30 min in 0.8 ml of charcoal buffer (10% activated charcoal in 0.02 M Tris–HCl buffer at pH 7.5) and shaken for 30 min. The samples were subjected to centrifugation at 19 000 g for 15 min and 0.2 ml of the supernatant was counted in a liquid scintillation counter (WinSpectral 1414, Wallac).

**Statistical analyses**

All data are presented as mean ± S.E.M. Metabolic data from the electroporated C57BL/6J mice and skeletal muscle gene expression from the exercise training cohort were analyzed using a paired Student’s t-test. Differences in gene expression in C2C12 cells, lean vs ob/ob mice, and normal glucose tolerant vs type 2 diabetic participants were analyzed using Student’s t-test after natural log transformation. Palmitate oxidation and glycogen synthesis in C2C12 myoblasts were analyzed using a two-way ANOVA, followed by Bonferroni’s correction for multiple comparison. The analyses were performed using GraphPad version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons were considered to be statistically significant at \( P < 0.05 \).

**Results**

**Overexpression of Twist1 or Twist2 in mouse tibialis anterior muscle**

Tibialis anterior muscle of C57BL/6J mice was electroporated with a vector containing an optimized sequence for either Twist1 or Twist2, or a control empty plasmid into the contralateral leg. While overexpression did not affect
the mRNA level of endogenous *Twist*, the exogenous transcript was overexpressed 3.3 times for *Twist1* and 3.5 times for *Twist2* compared with the contralateral muscle receiving the control plasmid (Fig. 1A).

mRNA levels of a number of gene targets of *Twist* were determined using RT-PCR. As expected from previous investigation (Pan et al. 2009), overexpression of *Twist1* and *Twist2* tended to reduce *PGC1α* mRNA expression (−36% ± 0.06 s.e.m. (*P* = 0.08) when overexpressing *Twist1* and −32% ± 0.05 s.e.m. (*P* = 0.001) when overexpressing *Twist2*). The early differentiation marker *Pax7* was increased following *Twist1* overexpression in skeletal muscle (Fig. 1B), and intermediate and late differentiation markers *MyoD* and *myogenin* trended in the same direction. *TWIST2* overexpression did not alter the expression of these genes (Fig. 1B). *Pdk4* mRNA was reduced by 26 and 38% following *TWIST1* or *TWIST2* overexpression respectively (*P* < 0.05). mRNA levels of inflammatory cytokines IL6, IL1β, and TNFα were increased by more than 60% (Fig. 1B and C, *P* < 0.05).

A modified oral glucose tolerance test including an injection of radiolabeled 2-deoxy-D-glucose was carried out in mice 1 week after electroporation. Total radioactivity in tibialis anterior muscle harvested 2 h after a bolus glucose injection was measured. Glucose uptake was unaltered in skeletal muscle overexpressing either *TWIST1* or *TWIST2* vs the respective contralateral control muscle (Fig. 1D). In contrast, total glycogen content was decreased by 39 and 28% in tibialis anterior muscle overexpressing *TWIST1* and *TWIST2* respectively (Fig. 1E, *P* < 0.05).

Overexpression of *TWIST2* was confirmed at the protein level in the leg electroporated with *Twist2* plasmid as compared with the control leg (Fig. 2). We were unable to obtain an antibody to reliably detect *TWIST1* protein by western blot analysis, despite trying three different sources (see section Materials and methods). As *TWIST1* and *TWIST2* overexpression reduced skeletal muscle glycogen content, despite similar rates of glucose uptake, we measured GS and its inactive phosphorylated fraction (serine 641). However, GS and phospho-GS were unaltered in the skeletal muscle overexpressing *TWIST2*. In contrast, both total GS protein and its phosphorylated fraction were reduced by 40% by overexpression of *TWIST1* (Fig. 2). We determined ACC content and ACC phosphorylation on serine 79. Overexpression of *TWIST1* or *TWIST2* in skeletal muscle resulted in a reduction in total ACC, without altering the level of phosphorylated protein, suggesting a larger fraction of total ACC in an inactive state (Fig. 2). We also measured AKT and AKT phosphorylation on serine 473. Total AKT protein levels were unchanged by TWIST overexpression, whereas AKT phosphorylation was increased by 2.5-fold by TWIST1 and 1.9-fold by TWIST2 overexpression.

**Effect of TWIST1 or TWIST2 overexpression in C2C12 myoblasts**

Overexpression of *TWIST1* or *TWIST2* was observed in cultured C2C12 mouse myoblasts. The experiments were...
Figure 2
Western blotting analysis carried out in paired mouse skeletal muscle overexpressing TWIST1 (n = 5) or TWIST2 (n = 6). TWIST2, GS, p-GS, ACC, p-ACC, AKT, p-AKT levels were measured. Their respective phosphorylation ratio was calculated. Protein levels were normalized for GAPDH. (A) Representative blots of samples electroporated with TWIST1. (B) Relative protein level and phosphorylation ratio for samples electroporated with TWIST1. (C) Representative blots of samples electroporated with TWIST2. (D) Relative protein level and phosphorylation ratio for samples electroporated with TWIST2. Results are mean ± S.E.M. **P<0.01; *P<0.05. o.e., overexpression.
carried out in myoblast cultures because TWIST1 has been shown to lead to dedifferentiation in C2C12 myotubes (Hjiantoniou et al. 2008). Overexpression of TWIST2 increased mRNA levels by more than tenfold ($P<0.05$, Fig. 3A). Confirming studies reported by other investigators (Hebrok et al. 1997, Murakami et al. 2008), Twist1 mRNA was undetected in cultured C2C12, and thus the overexpression that Twist1 achieved could not be related with endogenous levels. Nevertheless, the overexpression Ct values achieved were similar for Twist1 and Twist2 (24.2 Ct for Twist1 and 25.0 Ct for Twist2 respectively). Similar to the results achieved following the in vivo overexpression, we noted a 30% reduction in Pdk4 mRNA levels and an increase of more than 60% in Il6 mRNA levels (Fig. 3B and C) in cultured myoblasts 24 h after transfection.

We determined the media appearance of Il6, Il1β, and Tnfα in 16-h conditioned media from C2C12 myoblasts. IL6 and TNFα were not detectable under any conditions investigated. The media content levels of IL1β were not significantly altered by overexpression of TWIST1 or TWIST2 (29±7 pg/ml for control, 22±4 pg/ml for Twist1, and 27±8 pg/ml for Twist2 respectively, NS)

Glucose incorporation into glycogen and lipid oxidation was determined in myoblasts overexpressing TWIST1 or TWIST2. Insulin increased glucose incorporation by 40% in control myoblasts. However, overexpression of either Twist1 or Twist2 did not alter either basal or insulin-stimulated glycogen synthesis (Fig. 4A). Moreover, palmitate oxidation was unaltered in the myoblasts overexpressing either Twist1 or Twist2 (Fig. 4B).

### Effects of obesity, T2D, or exercise training on Twist expression

The mRNA levels of Twist1 and Twist2 were determined in tibialis anterior muscle from insulin resistant ob/ob mice and lean WT mice. As expected ob/ob mice were heavier than their lean counterparts (55.5 g±2.2 for ob/ob vs 20.7±0.5 for WT, mean±s.e.m., $P<0.001$) but had not significantly different levels of blood glucose level (13.0±4.5 s.e.m. for the ob/ob vs 7.9±0.3 s.e.m. for the WT, $P=0.27$). Although a tendency for reduced Twist1 mRNA was noted in tibialis anterior muscle from ob/ob mice ($P=0.085$), mRNA levels of either Twist1 or Twist2 tibialis anterior were not significantly altered (Fig. 5A). Likewise, TWIST1 mRNA was unaltered in the skeletal muscle from type 2 diabetic patients vs normal glucose tolerant control subjects (Fig. 5B). Finally, the mRNA level was determined in skeletal muscle biopsies from young healthy people before and after a 3-week exercise training program. Although exercise training improved maximal oxygen uptake and other clinical features (Czepluch et al. 2011), mRNA expression of TWIST1 or TWIST2 was unaltered (Fig. 5B).
TWIST1 and TWIST2 expression is altered in adipose tissue in the context of metabolic disease and implicated in the regulation of cytokine expression (Sosic et al. 2003, Pan et al. 2009, Pettersson et al. 2010, 2011). We hypothesized that TWIST proteins play a similar role in skeletal muscle. To this end, we induced overexpression of TWIST1 or TWIST2 in tibialis anterior muscle of C57BL/6J mice and cultured mouse C2C12 skeletal muscle myoblast cells. We also determined the effects of obesity, T2D, and exercise training on mRNA levels of TWIST1 and TWIST2 in skeletal muscle from different cohorts. Taken together, while our data support a role for TWIST proteins in metabolic processes, TWIST1 or TWIST2 levels in skeletal muscle are not altered in metabolic disease.

We determined the effect of TWIST1 or TWIST2 overexpression in intact mouse muscle and C2C12 myoblasts. As revealed by RT-PCR, TWIST1 overexpression in differentiated muscle increased the expression of Pax7, a gene involved in early-stage differentiation and an inducer of MyoD transcription (Hu et al. 2008). In line with the increase in Pax7, MyoD mRNA expression displayed a similar trend (P=0.07). Nevertheless, TWIST1 is a known inhibitor of MYOD transactivation (Hamamori et al. 1997) and its overexpression is likely to block any possible effect of an increase in MYOD. Furthermore, mRNA levels of Myogenin revealed a similar trend to MyoD. Interestingly, these three mRNA levels were not altered in skeletal muscle following TWIST2 overexpression, highlighting a specific difference in gene regulation between TWIST1 and TWIST2. The noted changes in mRNA levels of genes important for differentiation in TWIST1-overexpressing skeletal muscle could be due to an increase in cells (re)-entering cell-cycle as a compensation to the de-differentiation stimulus induced by TWIST1 (Hjiantoniou et al. 2008). The lack of change in Pax7 in response to TWIST2 overexpression could reflect the fact that TWIST2 is not critical in the differentiation process of skeletal muscle cells as whole-body knock-out mice are born with similar skeletal muscle mass as WT animals (Sosic et al. 2003).

We determined mRNA expression of several inflammatory cytokines. Our findings were consistent with previous results in human primary white adipocytes whereby TWIST1 silencing reduces mRNA expression of IL6 and TNFα (Pettersson et al. 2010). In contrast, both whole-body knock-out of TWIST2 or siRNA gene silencing of TWIST1 when coupled with TNFα stimulation in cultured adipocytes increases the expression of inflammatory cytokines (Sosic et al. 2003, Pettersson et al. 2011). Thus overexpression and ablation have similar effects, which may initially appear contradictory. However, as TWIST is a bHLH transcription factor, overexpression could have similar effects to silencing where homodimers have opposing effects to those mediated by heterodimers (Firulli et al. 2007, Connerney et al. 2008). Thus, the effects of TWIST proteins on cytokine expression appear to have a U-shaped curve with both extremes triggering a pro-inflammatory response.

We attempted to quantitate media appearance of IL-6 and TNFα secretion in C2C12 myoblasts, but these were below the assay detection limit. Secretion of TNFα and IL6 in C2C12 myoblasts has previously been reported to be close to the detection limit (Chen et al. 2007) or only detectable after 10 ng/ml epinephrine stimulation (Frost et al. 2004). While media content of IL1β was detectable, no significant difference was noted in the expression of this cytokine following overexpression of either TWIST1 or TWIST2. Whether the increased mRNA levels of these cytokines following overexpression of

![Figure 4](image-url)

**Figure 4**

Glycogen synthesis and palmitate oxidation in C2C12 mouse myoblasts overexpressing TWIST1 or TWIST2. (A) Glycogen synthesis; white bars: basal. Black bars: after 2 h stimulation with 120 nM insulin. Data are representative of four independent experiments. Results are representative of three independent experiments. Results are mean ± S.E.M of fold of control. **P<0.01; *P<0.05. o.e., overexpression.

**Discussion**

The noted changes in mRNA levels of genes important for differentiation in TWIST1-overexpressing skeletal muscle could be due to an increase in cells (re)-entering cell-cycle as a compensation to the de-differentiation stimulus induced by TWIST1 (Hjiantoniou et al. 2008). The lack of change in Pax7 in response to TWIST2 overexpression could reflect the fact that TWIST2 is not critical in the differentiation process of skeletal muscle cells as whole-body knock-out mice are born with similar skeletal muscle mass as WT animals (Sosic et al. 2003).

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![Figure 5](image-url)

**Figure 5**

TWIST1 and TWIST2 mRNA expression in obesity, T2D, or following exercise training. (A) TWIST1 and TWIST2 expression in ob/ob mice skeletal muscle (n=10). (B) TWIST1 expression in human vastus lateralis muscle from NGT and T2D subjects. (C) TWIST1 and TWIST2 expression in human vastus lateralis muscle of healthy subjects before and after 3 weeks of endurance training (n=13). Results are mean ± S.E.M. of fold of control.
either TWIST1 or TWIST2 would modulate the secretion of differentiated or TNFα-stimulated cells remains to be determined. In a previous study in adipocytes, TWIST1 affected MCP1 but not IL6 secretion following stimulation with TNFα (Pettersson et al. 2011).

Whole-body-targeted ablation of Twist2 in mouse models decreases glycogen stores (Sosic et al. 2003). However, using electroporation-mediated gene transfer, we note that overexpression of TWIST2 depleted muscle glycogen content. These results, although paradoxical, are consistent with the hypothesis that TWIST has analogous effects when ablated or overexpressed. Despite reduced glycogen stores in muscle overexpressing Twist, glucose uptake was unaltered. Based on this observation, we hypothesized that the glucose flux into the cell may be shifted toward the Krebs cycle. Thus, we determined Pdk4 expression because this enzyme is the master regulator of pyruvate dehydrogenase in skeletal muscle (Sugden & Holness 2006). The reduced Pdk4 mRNA expression noted following TWIST overexpression is consistent with an activation of pyruvate dehydrogenase and increased glucose flux into the Krebs cycle, rather than glycogen synthesis. Our results are further supported by a reduction in the total and active (nonphosphorylated) GS in TWIST1-overexpressing skeletal muscle. Whether glyco-
gen phosphorylase or de-branching enzyme activity was altered in TWIST-overexpressing muscle was not assessed in the current study; however, neither basal nor insulin-stimulated glycogen synthesis was altered in TWIST-overexpressing myoblasts. Taken together, our data suggest that overexpression of TWIST1 or TWIST2 results in an increased rate of glycogen utilization.

Overexpression of TWIST1 or TWIST2 in intact mouse muscle resulted in a reduced ACC protein content, which could indicate a decreased fatty acid synthesis capacity. Furthermore, the total level of the phosphorylated form of ACC was unchanged, leading to a larger ratio of inactivated ACC. In cultured C2C12 cells overexpressing TWIST1 or TWIST2, fatty acid oxidation in vitro was unchanged. Thus if glucose utilization is increased while fatty acid oxidation is unaffected, total energy probably increased. This for example could be reflected by an increase in inflammation, as suggested by the increase in inflammatory cytokine transcripts. Cell growth is also an energy-requiring process in which a role for TWIST proteins has been implicated (Shiota et al. 2008, Isenmann et al. 2009). In line with this, AKT Ser473 phosphorylation was increased in TWIST1 or TWIST2-overexpressing muscles 2 h after a glucose challenge. Activation of AKT is closely linked to cell growth and cell survival (Jacinto et al. 2006), two energy-demanding processes. Thus, overexpression of TWIST1 in skeletal muscle reduces glycogen content, while glucose uptake and glycogen synthesis appear unaffected. As fatty acid oxidation is also unchanged, glycogen stores may be utilized for energy-dependent processes partly induced by AKT activation. These results underpin a role of TWIST in facilitation of glucose utilization, which is a key feature in cancer development (Warburg 1956).

We also determined whether TWIST expression in skeletal muscle is altered due to obesity, T2D, or exercise training. While TWIST1 has been previously reported to be undetectable in skeletal muscle (Pettersson et al. 2010), our analysis in human as well as mouse skeletal muscles indicates that there is a consistent and detectable amount of the transcript, which is in agreement with other available expression data (Roth et al. 2006, Hagg et al. 2009). Nevertheless, skeletal muscle TWIST mRNA levels were unaltered by obesity in ob/ob mice, as well as in patients with T2D or after exercise training in healthy people. Thus, alterations in TWIST expression in skeletal muscle do not account for changes in insulin sensitivity in obesity, T2D, or exercise training.

TWIST1 has been implicated in the regulation of IL6 in adipose tissue. As skeletal muscle is also a source of IL6 (Steensberg et al. 2000), we reasoned that TWIST1 may impact IL6 expression in skeletal muscle. ob/ob mice present an extreme obesity phenotype and have higher circulatory levels of IL6 (Harkins et al. 2004). However, as noted above, if anything, Twist1 expression tended to be reduced in skeletal muscle from obese mice, consistent with data associating a reduced Twist1 expression with an increased Il6 expression in adipose tissue (Pettersson et al. 2011). Furthermore, an endurance exercise bout is associated with acute IL6 production (Drenth et al. 1995). While TWIST1 and TWIST2 mRNA levels were unaltered following exercise training, the biopsy was obtained 48 h after the last bout of exercise, when the plasma IL6 concentration returns to baseline level (Ostrowski et al. 1998). Whether exercise training leads to an acute and transient change in TWIST expression remains to be determined. Taken together, the data presented in this study provide evidence that IL6 expression in skeletal muscle is regulated via TWIST1 and TWIST2, but whether acute changes in IL6 expression are dependent on TWIST is currently not known.

In summary, we show that overexpression of TWIST1 or TWIST2 in skeletal muscle increases inflammatory gene expression, reduces glycogen content, promotes glucose utilization and cell growth pathways while reducing cell
capacity for fatty acid synthesis but not for fatty acid oxidation. Skeletal muscle expression of TWIST was unaffected by altered metabolic contexts, such as obesity, T2D, or exercise training. Thus, while TWIST proteins are important for skeletal muscle development and alter glucose utilization and glycogen stores in differentiated mature muscle, our data indicate that TWIST expression in skeletal muscle is not altered with metabolic disease, underlining that the role of TWIST is differentiation, tissue, and context dependent.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
J M M performed the experiments, analyzed and researched data, and wrote the manuscript. F L M S and J M analyzed and researched data, contributed to discussion, reviewed and edited the manuscript. A K designed the study, analyzed data, contributed to discussion, wrote manuscript, and reviewed and edited the manuscript.

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