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ORIGINAL ARTICLE

The *Ski* proto-oncogene regulates body composition and suppresses lipogenesis

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Objective: The *Ski* gene regulates skeletal muscle differentiation *in vitro* and and *in vivo*. In the *c-Ski* overexpression mouse model there occurs marked skeletal muscle hypertrophy with decreased adipose tissue mass. In this study, we have investigated the underlying molecular mechanisms responsible for the increased skeletal muscle and decreased adipose tissue mass in the *c-Ski* mouse.
Approach: Growth and body composition analysis (tissue weights and dual energy X-ray absorptiometry) coupled with skeletal muscle and white adipose gene expression and metabolic phenotyping in *c-Ski* mice and wild-type (WT) littermate controls was performed.
Results: The growth and body composition studies confirmed the early onset of accelerated body growth, with increased lean mass and decreased fat mass in the *c-Ski* mice. Gene expression analysis in skeletal muscle from *c-Ski* mice compared with WT mice showed significant differences in myogenic and lipogenic gene expressions that are consistent with the body composition phenotype. Skeletal muscle of *c-Ski* mice had significantly repressed Smad1, 4, 7 and myostatin gene expression and elevated myogenin, myocyte enhancer factor 2, insulin-like growth factor-1 receptor and insulin-like growth factor-2 expression. Strikingly, expression of the mRNAs encoding the master lipogenic regulators, sterol-regulatory enhancer binding protein 1c (SREBP1c), and the nuclear receptor liver X-receptor-α, and their downstream target genes, *SCD-1* and *FAS*, were suppressed in skeletal muscle of *c-Ski* mice, as were the expressions of other nuclear receptors involved in adipogenesis and metabolism, such as peroxisome proliferator-activated receptor-γ, glucocorticoid receptor and retinoic acid receptor-related orphan receptor-α. Transfection analysis demonstrated Ski repressed the SREBP1c promoter. Moreover, palmitate oxidation and oxidative enzyme activity was increased in skeletal muscle of *c-Ski* mice. These results suggest that the Ski phenotype involves attenuated lipogenesis, decreased myostatin signalling, coupled to increased myogenesis and fatty acid oxidation.
Conclusion: *Ski* regulates several genetic programs and signalling pathways that regulate skeletal muscle and adipose mass to influence body composition development, suggesting that *Ski* may have a role in risk for obesity and metabolic disease.
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Introduction

The *c-Ski* proto-oncogene has major roles in controlling cell proliferation and skeletal muscle differentiation *in vitro* and *in vivo*.[1–3] Originally cloned from acute transforming retroviruses through homology to the viral oncogene *v-Ski*,[4] *c-Ski* functions as an oncogenic protein and induces myotube formation of quail embryonic fibroblasts.[5] Transgenic mice that overexpress the *c-Ski* gene display skeletal muscle hypertrophy and by contrast, a marked decrease in adipose

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tissue mass.[2] The dramatic increase in skeletal muscle mass in the *c-Ski* transgenic mouse model involves selective hypertrophy of the type IIb 'fast twitch' fibres. The slow twitch oxidative type 1 and IIa fibres do not appear to be enlarged.[2,6]

Ski is a repressor of the transforming growth factor-β (TGF-β) signalling pathway and interacts directly with the signal transduction proteins, Smad1-4 in the nucleus.[7–10] The TGF-β signalling family consists of multifunctional proteins that regulate the growth, differentiation, apoptosis and morphogenesis of a wide variety of cells.[11] In addition, a ligand belonging to the TGF-β superfamily, myostatin, has been described as a potent negative regulator of skeletal muscle growth and promoter of adipogenesis.[12,13] Skeletal muscle differentiation requires the action of two groups of regulatory transcription factors: muscle regulatory factors of the MyoD family (muscle-specific β-helix–loop–helix proteins) including MyoD and its relatives Myf5, myogenin and muscle regulatory factor 4, and the myocyte enhancer factor 2 (MEF2_MADS (MCM1, agamous, deficiens, serum reponse factor) box proteins) family that includes myocyte enhancer factor 2 (MEF2) and E proteins.[14] The muscle regulatory factors with MEF2 family proteins coordinate the action of many different co-activators and co-repressors and signalling proteins (for example, p38 kinase) to control myogenesis. Nuclear Ski enhances muscle differentiation through modulation of myogenin promoter activity[15] through activation of the MyoD–myocyte enhancer factor 2 (MEF2) complex and inhibition of histone deacetylase activity.[16] Ski has also been reported to activate the enhancer/promoter of myosin light chain 1/3 and muscle creatine kinase.[17]

In addition to its role in TGF-β signalling, Ski acts as a repressor of a diverse range array of other transcription factor signalling pathways. These include nuclear hormone receptors (NRs), such as retinoic acid receptor[18,19] and thyroid hormone receptor with a Mad–histone deacetylase complex,[20] and transcription factors involved in erythroid and macrophage differentiation, such as PU.1 and GATA1, respectively.[20–22]

Although several studies have examined the physiological effects of skeletal muscle hypertrophy in the Ski transgenic mouse, almost nothing is known about the mechanisms that mediate the decreased adiposity of these mice and whether Ski alters skeletal muscle metabolism or metabolic gene expression in target tissues, such as skeletal muscle or adipose tissue.[2] Moreover, although *in vitro* studies have identified several muscle-specific differentiation transcription factors that Ski regulates in the β-helix–loop–helix and MADS box protein family, such as MyoD and myogenin,[15,16] the role of c-Ski in modulation of other gene targets known to be involved in skeletal muscle hypertrophy has not been examined.

Skeletal muscle accounts for ~40% of the total body mass and energy expenditure. Moreover, it is very dependent on fatty acid and glucose oxidation to meet its energy demands. Consequently, this organ has a significant role in lipid, glucose and energy homeostasis. The decreased adiposity of *c-Ski* mice, may involve the onerous metabolic demands of the significantly larger lean tissue mass. We hypothesize that Ski-mediated skeletal muscle hypertrophy alters the balance between fatty acid oxidation and lipogenesis to meet the altered metabolic demands of this mouse model.

In this study, we have re-evaluated the growth pattern and body composition in the *c-Ski* mouse and correlated these physiological findings to changes in gene expression (controlling metabolism) and several metabolic parameters in skeletal muscle and adipose tissue. We show that the *c-Ski* mice have an altered gene expression footprint that may explain the lean and hypertrophic phenotype. Strikingly, skeletal muscle of *c-Ski* mice display concordant changes in several genetic programs that include increased muscle mass, decreased adiposity and suppressed lipogenesis. Moreover, *c-Ski* mice seem to have increased fatty-acid β-oxidation. These results suggest that Ski targets several signalling pathways that promote increased muscle mass and decreased adiposity. This mouse model provides insights into potential pathways that may be therapeutically modulated in lean tissue to treat obesity and metabolic disease.

Materials and methods

Animals and tissues collection

The *c-Ski* transgenic mice (line 8566) that express chicken *c-Ski* cDNA under control of the murine sarcoma virus long terminal repeat (MSV, LTR) mouse were originally obtained from Stephen Hughes (NIH, Bethesda, MD, USA)[23] and the colony was maintained by back-crossing with C57Bl/6JArc for >10 generations. Genotyping was performed using PCR. The colony was housed at the vivarium of the Children's Medical Research Institute (CMRI) in Sydney with light:dark cycle of 12:12 h (7am on: 7pm off). The mice were fed a standard rodent pelleted diet (19.6% protein, 4.6% fat, 4.3% crude fibre, 14.3 MJ kg^{-1} digestible energy) purchased from Glen Forest Speciality Feeds (Glen Forrest, WA, Australia) and given water *ad libitum*. Experimental animals were weighed weekly from weaning to 15 weeks of age to determine the growth curve. At 15 weeks of age, male mice were transferred into another cage and given access to unlimited supply of water for an overnight fast (14–16 h). Blood collection was performed through cardiac puncture under isoflurane anaesthesia and then animals killed by cervical dislocation. Tissues were then collected and stored in RNA later (Sigma-Aldrich, St Louis, MO, USA) and treated according to the manufacturer's protocol. All animal studies were approved by the Children's Medical Research Institute and The University of Queensland Animal Ethics Committees.

Growth curve analysis

Data on 47 mice with 288 observations were analysed. Models predicting mouse weight over time for each mouse genotype were developed using multilevel mixed effects linear

regression. A quadratic term was incorporated into the models to account for the non-linear relationship between weight and time. The variables time, time squared and sex were modelled as fixed effects, whereas the intercept and slope of the growth curve were modelled as random effects to account for the varying start weights and growth rates. The coefficients for each parameter in the models were then compared. To explore the difference in growth rates between wild and transgenic mice, an overall model was developed similar to those previously described, however, this model incorporated the variable mouse genotype and an interaction term. The purpose of this was to determine whether mouse genotype had a significant interaction effect with any of the other predictors of weight. The interaction term and mouse genotype were modelled as fixed effects and the regression model was re-run with each of the following interaction terms: mouse genotype and sex or time or time^2. The statistical package Stata 10.1 for Windows (StataCorp LP, College Station, TX, USA) was used to perform the growth curve analysis.

Food intake

The protocol developed by the Jackson Laboratories (Bar Harbor, ME, USA) was used. Food intake was determined to the nearest 0.1 g by weighing the metal cage top containing the food each day. In previous tests, using this procedure we have shown that food spillage does not exceed 0.1 g day^{-1} per mouse. Total food intake of 15-week-old mice was measured per mouse box (2–3 mice of the same genotype per box) for five consecutive days to obtain average daily food intake per mouse.

Histology

Tissues were fixed in 10% buffered formalin, paraffin-embedded and sections of liver, brown and white adipose tissues were stained with hematoxylin and eosin.

Dual energy X-ray absoptiometry and tissues weight analysis

Whole animals were placed in a Mouse GE Lunar PIXImus Densitometer (GE Healthcare, Piscataway, NJ, USA) to determine total body fat and lean tissue composition as described previously.[24] Tissue weights were determined on 14–16 h fasted mice after cervical disslocation.

Quantitative RT–PCR

TaqMan low density array cards (while adipose tissue) were analysed on an ABI Real Time 7900 (Applied Biosystems, Forster City, CA, USA) according to the manufacturer protocol. All RT–PCR assays were performed on the ABI Real Time 7500 thermocycler using the following temperatures and cycles: one cycle of 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. For SYBR-based assays (Applied Biosystems, Forster City, CA, USA), a dissociation stage was performed for one cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Primers for qRT–PCR analysis of the mRNA populations using SYBR green have been described in detail for 18S, sterol-regulatory enhancer binding protein 1c (*SREBP1c*), stearoyl-CoA desaturase-1 (*SCD-1*), fatty acid synthase (*FAS*), myogenin,[25] peroxisome proliferator-activated receptor-γ (*PPAR*γ),[25] and liver X-receptor-α (*LXR*α).[26] Following SYBR primers were designed using Primer Express (Applied Biosystems, Foster City, CA, USA): *c-Ski* (F:5′-GAAAAGCAG TCCAGTTGGTTACG-3′; R:5′-ATGGACACAGC CAATGCTCTTA-3′); activin receptor IIB (F:5′-ACGTGGCGGA GACGATGT-3′; R:5′-GTGAGGTCGCTCTTCAGCAGTAC-3′); growth hormone receptor (F:5′-ATTCACCAAGTGTCGTTCC CC-3′; R:5′-TGCAGCTTGTCGTTGGCTT-3′); insulin growth factor-1 (F:5′-TGGTGGATGCTCTTCAGTTCG-3′; R:5′-AGCTC CGGAAGCAACACTCAT-3′); insulin growth factor-1 receptor (F:5′-AGCACCCAGAGCATGTACTGTATC-3′; R:5′-GAGCAG AAGTCACCGAATCGA-3′); Interleukin-15 (F:5′-TGAGGCTGG CATTCATGTCTT-3′; R:5′-CATCTATCCAGTTGGCCTCTGTT-3′). Assay-on-Demand TaqMan primer/probe sets were used to assay expression of: *Cox-2* (Mm00478374_m1), glucocorticoid receptor (*GR*) (Mm00433832_m1), insulin-like growth factor-2 (*IGF-2*) (Mm00439564_m1), *MyoD* (Mm00440387_m1), *MEF2c* (Mm01340839_m1), Myostatin (Mm00440328_m1), *ROR*α (Mm00443103_m1), *Ski* (Mm00448744_m1), *Smad2* (Mm00487530_m1), *Smad3* (Mm00489637_m1), *Smad4* (Mm00484724_m1), *Smad6* (Mm00484738_m1), *Smad7* (Mm00484741_m1).

RNA extraction and cDNA synthesis

Total RNA from liver, skeletal muscle and pancreas was extracted using TRI reagent and a handheld ultra-turrax homogenizer (IKA, Staufen, Germany) and purified using the RNeasy mini kit (Qiagen, Valencia, CA, USA). The same procedure was used for white and brown adipose tissues with the exception that Qiazol was used instead of TRI reagent. Reverse transcription was performed using 1 μg of total RNA in each cDNA synthesis reaction.[25]

Blood and serum analysis

Glucose tolerance tests (2 g kg^{-1} glucose i.p.) were performed in overnight-fasted mice. Blood samples were obtained from the tail tip at the indicated times, and glucose levels were measured using a glucometer (AccuCheck II; Roche, Basel, Switzerland). Insulin was assayed by radioimmunoassay (Linco Research, St Louis, MO, USA). The concentrations of non-esterified fatty acids (Wako Pure Chemical Industries, Osaka, Japan) and triglycerides (Roche Diagnostics) were determined using a colorimetric kit. Serum levels of IGF-1, IGF-2, leptin and adiponectin were measured using Milliplex kits (Millipore, St Charles, MO, USA) and a Mediagnost Kit according to the manufacturer's protocol.

Homogenate oxidations

Palmitate oxidation were measured in muscle homogenates using a modified method described previously.[27,28] Briefly,

muscles were homogenized in 19 volumes of ice-cold 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris–HCl, and 1 mmol l^{-1} EDTA (pH 7.4). For assessment of palmitate oxidation, 50 μl of muscle homogenate was incubated with 450 μl reaction mixture (pH 7.4). Final concentrations of the reaction mixture were (in mmol l^{-1}): 100 sucrose, 80 KCl, 10 Tris–HCl, 5 KH_2PO_4, 1 $MgCl_2$, 2 malate, 2 ATP, 1 dithiothreitol, 0.2 EDTA, 2 L-carnitine, 0.05 coenzyme A, 0.3% fatty acid-free BSA and 0.2 [1-^{14}C]palmitate (0.5 μCi). After 90 min of incubation at 30 °C, the reaction was stopped by the addition of 100 μl of ice-cold 1 mol l^{-1} perchloric acid. Carbon dioxide produced during the incubation was collected in 100 μl of 1 mol l^{-1} sodium hydroxide. The ^{14}C counts present in the acid-soluble fraction were also measured and combined with the CO_2 values to give the total palmitate oxidation rate. Protein content in the homogenates were measured using the Bradford method (Protein Assay Kit; Bio-Rad Laboratories, Regents Park, Australia).

Enzyme activity measurements

Powdered muscle samples were homogenized 1:19 (wt/vol) in 50 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA and 0.1% Triton X-100 (pH 7.2), using a Polytron instrument (Kinematica, Littau-Lucerne, Switzerland) and were subjected to three freeze–thaw cycles. Cytochrome *c* oxidase, citrate synthase and β-hydroxyacyl CoA dehydrogenase levels were determined at 30 °C, as described previously,[28] using a Spectra Max 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

C2C12 cell culture, transfection and luciferase assay

The effect of Ski on SREBP1c promoter activity was tested by transiently transfecting C2C12 cells with a luciferase reporter plasmid (pGL3-SREBP1c)[29] and a *c-Ski* expression plasmid (pCS2 + MT-cSki; a kind gift of M. Hayman, Stony Brook University, NY, USA). Each well of a 24-well plate of C2C12 cells (30% confluence) was transfected with a total of 0.5 μg of DNA consisting of 0.3 μg pGL3-SREBP1c reporter and either 0, 10, 50, 100 or 200 ng of pCS2 + MT-cSki with total pCS2 + MT plasmid DNA made up to 200 ng with pCS2 + MT (empty vector). A liposome-mediated transfection procedure was used as previously described.[25] Cells were transfected using a DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulphate and metafectene (Biontex Laboratories GmbH, Munich, Germany) liposome mixture in 1 × HEPES-buffered saline (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na_2HPO_4, 11 mM dextrose (pH 7.1)). The DNA–DOTAP–metafectene mixture was added to the cells in 0.6 ml of Dulbecco's modified Eagle's medium supplemented with 5% foetal calf serum. After 16 h the culture medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and either vehicle (0.1% dimethyl sulphoxide) or LXR agonist (1 μM T0901317). After a further 48 h, the cells were collected for the measurement of luciferase activity. Luciferase activity was assayed using a Luclite kit (Packard, Meriden, CT, USA) and Wallac Trilux 1450 Microbeta Luminometer (Perkin Elmer, Walham, MA, USA), according to the manufacturer's instructions. For each experiment, conducted in triplicate, the relative light units were normalized to the vehicle-treated, pCS2 + MT empty vector-transfected control. The normalized data mean ± s.e.m. from three independent experiments were plotted.

Statistical analysis

Unless stated otherwise, all data were analysed using a *t*-test on GraphPad Prism 4 software package (La Jolla, CA, USA).

Results

Ski *transgenic mice have early post-natal enhancement of growth with increased lean mass and decreased fat mass*

Although, previous studies of the *c-Ski* mice have shown altered skeletal muscle mass with profound enlargement of specific skeletal muscle types involving type II fibres, a detailed examination of the alterations in adipose tissue mass in the *c-Ski* mice have not been previously undertaken.[2]

We confirmed the findings of previous studies of the *c-Ski* mouse model by performing a growth analysis of both male and female Ski and wild-type (WT) littermate mice. To the best of our knowledge such a detailed growth curve has not been previously published. *c-Ski* mice have early post-natal enhancement of body weight that is readily apparent by 6–8 weeks of age when both male and female *c-Ski* mice are significantly heavier than WT littermate mice (Figure 1a). By maturity at 15 weeks of age, the *c-Ski* mice have a 25–30% increase in body weight compared with their WT controls. The change in body size was accompanied by a significant increase in food intake in *c-Ski* mice compared with WT mice (Figure 1b).

We performed a longitudinal growth curve analysis of WT and *c-Ski* mice using a multilevel mixed effects linear regression. The change in weight of the mice over time was found to be non-linear in all groups (males/females; transgenic/WT). Two models were developed predicting mouse weight using time, time squared and sex for each mouse genotype. Growth curves for *c-Ski* transgenic and WT mice were compared and found to be significantly different from each other (Table 1). A statistically significant interaction effect was observed between mouse genotype and time (Table 2). This confirmed that the coefficient for time is significantly different between WT and *c-Ski* transgenic mice.

Body composition studies using dual energy X-ray absorptiometry (Figures 1c and d) and analysis of organ weights (Figure 2a) confirmed that the increase in body weight in the Ski mice is associated with increase in size relative to body weight of muscles with predominately type 2 fibres (tibialis anterior and extensor digitorum longus) with no change in the type 1 fibre predominant soleus and mixed fibre-type

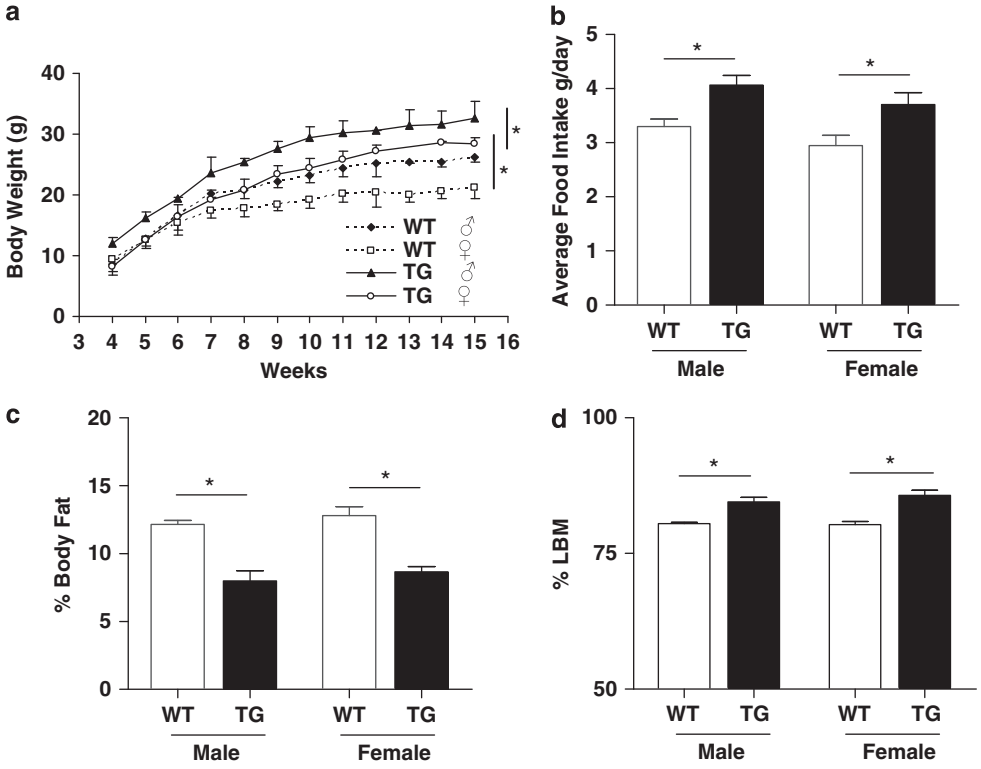


Figure 1 Overexpression of *c-Ski* in transgenic mice leads to early post-natal growth increase and altered body composition with increased lean and decreased body fat mass. (**a**) Total Body weight of *c-Ski* transgenic (TG; continuous line) and wild-type (WT; dashed line) mice from 4 to 15 weeks of age. Male mice (closed symbols) and female mice (open symbols) as indicated ($n=7$–15 males and females). *$P<0.05$ by analysis of variance (ANOVA) between sex-matched WT and TG mice. (**b**) Food intake between WT and TG male and female mice ($n=6$–8 mice in each group). Data are the average daily food intake per mouse over five consecutive days. (**c** and **d**) Body composition according to PIXImus in 15-week-old male and female WT and *c-Ski* (TG) mice ($n=5$–6 mice in each group). *$P<0.01$ by two-way ANOVA between sex-matched WT (white columns) and TG (black columns) mice. All data are presented as mean ± s.e.m.

Table 1 Longitudinal growth curve analysis of WT and *c-Ski* mice

Parameters	*Ski transgenic*				*Wild type*			
	Coefficient	*s.e.*	*95% CI*	P-*value*	*Estimate*	*s.e.*	*95% CI*	P-*value*
Intercept	−7.28	0.84	−8.94, −5.61	0.000	0.60	1.04	−1.44, 2.65	0.564
Time (weeks)	5.86	0.19	5.49, 6.22	0.000	3.58	0.15	3.27, 3.88	0.000
Time (weeks)^2	−0.22	0.01	−0.24, −0.20	0.000	−0.13	0.007	−0.15, −0.12	0.000
Sex (ref: males) females	−3.57	0.50	−4.55, −2.60	0.000	−3.48	0.39	−4.25, −2.72	0.000
Random effect parameters	*Estimate*	*s.e.*	*95% CI*		*Estimate*	*s.e.*	*95% CI*	
s.d. (time)	0.11	0.03	0.06, 0.20		0.45	0.07	0.34, 0.61	
s.d. (intercept)	0.13	0.27	0.001, 8.18		4.38	0.64	3.28, 5.83	
Corr (time, intercept)	0.99	0.02	−1, 1		−0.97	0.01	−0.99, −0.94	
s.d. (residuals)	0.94	0.07	0.80, 1.09		0.75	0.05	0.67, 0.85	
	Wald $\chi^2=2203.18$ $P=0.0000$				Wald $\chi^2=633.76$ $P=0.0000$			

Abbreviations: CI, confidence interval; WT, wild type.

gastrocnemius muscles. By contrast, all white and brown adipose tissue fat pads examined were decreased in size in the *c-Ski* mice compared with WT mice (Figure 2b). Interestingly, adipocytes from white and brown fat were significantly smaller in *c-Ski* mice than WT mice (Figure 2d). Notably, there was no change in liver size or the gross appearance of fat deposits in the liver of the *c-Ski* mice (Figure 2c). Overall these observations emphasize the

Table 2 Summary of model predicting mouse weight over time for WT and Ski mice (with statistically significant interaction term)

Parameters	*Effect*	*s.e.*	*95% CI*		P-*value*
Intercept	−2.72	1.18	−5.02	−0.41	0.021
Time (weeks)	5.35	0.26	4.85	5.86	0.000
Time (weeks)^2	−0.16	0.006	−0.18	−0.15	0.000
Sex (ref: male) female	−3.52	0.35	−4.20	−2.83	0.000
Type (ref: Ski) wild type	0.53	1.27	−1.96	3.03	0.675
Type*Time	−0.58	0.13	−0.84	−0.32	0.000
Random effects parameters	*Estimate*	*s.e.*	*95% CI*		
s.d. (time)	0.37	0.05	0.28	0.49	
s.d. (intercept)	3.59	0.50	2.72	4.72	
Corr (time, intercept)	−0.95	0.02	−0.98	−0.91	
s.d. (residuals)	0.93	0.05	0.84	1.02	

Abbreviations: CI, confidence interval; WT, wild type. Wald $\chi^2 = 1402.37$, *P*-value = 0.0000.

profound effect that Ski overexpression has on both muscle and adipose tissue mass in this mouse model.

Endogenous Ski *and ectopic* c-Ski *expression in transgenic mice:* c-Ski *specifically expressed in type II fibres*

To confirm whether *c-Ski* transgene expression had any effect on endogenous levels of *Ski* expression, we performed quantitative RT–PCR (qRT–PCR), using primers specific for murine *Ski* and *c-Ski*, on various tissues (Figure 3). When compared with WT mice there appeared to be no alteration in endogenous levels of *Ski* in the *c-Ski* TG mice in any of the tissues studied. Furthermore, this analysis confirmed that ectopic *c-Ski* expression is largely confined to Type II skeletal muscle fibres with levels of transgene expression highest in fast-fibre-predominant muscles (tibialis anterior and gastrocnemius) and lowest in the slow-fibre-predominant soleus muscle.[2] Interestingly, *c-Ski* transgene expression was also expressed at lower levels in white and brown adipose tissue and liver, whereas its expression was undetectable in pancreas (Figure 3).

Ectopic Ski *expression leads to repression of the TGF-*β *signalling gene expression*

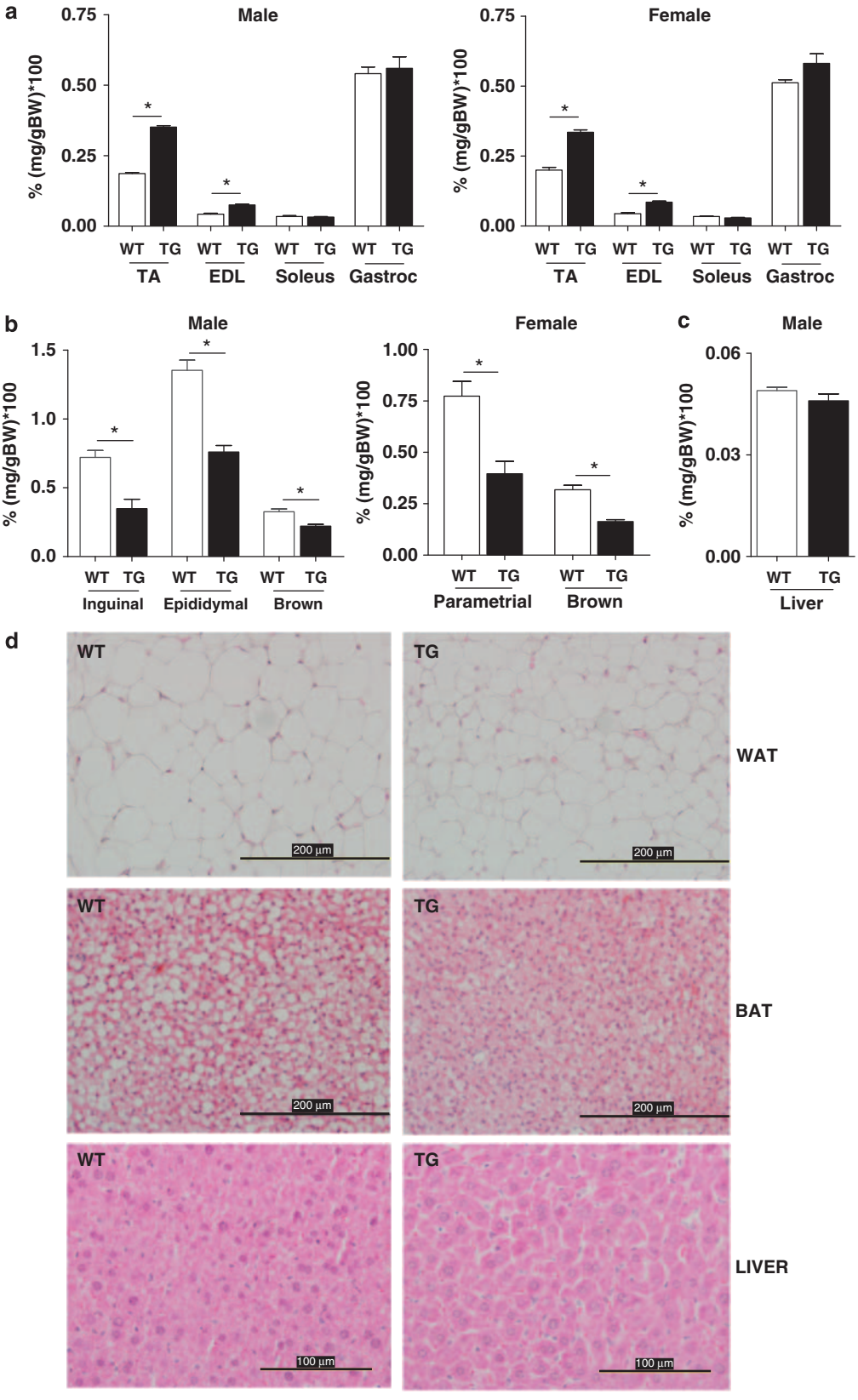
The mechanisms that mediate increased muscle mass and decreased adiposity in the *c-Ski* mice have not been investigated. To address this question, we first performed qRT–PCR expression analysis of genes that are known to regulate skeletal muscle mass. Ski is a major negative regulator of the TGF-β signalling pathway involved in cell proliferation. Accordingly, we examined the expression of several members of this pathway, including the Smad transducer proteins, the negative regulator of muscle mass, myostatin and the activin receptor type 2B (ACVR2B; Figure 4a). In *c-Ski* mice, the bone morphogenetic protein-specific Smad 1, and Co-Smad 4 and reverse Smad 7 were all significantly repressed compared with WT mice. There was a trend towards a decrease in expression of the TGF-β-specific Smads 2 and 3 and inhibitory Smad 6 and ACVR2B in *c-Ski* mice compared with WT mice, however, the changes did not attain significance (Smad2 $P = 0.14$, Smad3 $P = 0.08$, Smad 6 $P = 0.21$ and ACVR2B $P = 0.08$). In concordance with the increased muscle mass and decreased adiposity in the *c-Ski* mice, the skeletal muscle from *c-Ski* mice expressed significantly decreased levels of the mRNA encoding myostatin.[12,13]

Ectopic c-Ski *expression differentially regulates helix–loop–helix, MADs box and growth hormone receptor–IGF-1 gene expression*

Previous reports have demonstrated that Ski activates myogenin expression and promoter activity.[15] Consistent with these observations, we show that Ski skeletal muscle expresses markedly increased levels of the mRNA encoding myogenin relative to WT muscle (Figure 4b). Moreover, we show differential effects in the *c-Ski* mice on other skeletal muscle factors, such as *MEF2c* and *MyoD*, with opposite effects on expression patterns of these myogenic regulators.[30] Interestingly, Ski also seems to alter the expression of the growth hormone receptor and IGF signalling pathways, which have roles in skeletal muscle and adipose growth and development.[31–33] In the *c-Ski* mice, expression of insulin-like growth factor 1 receptor and IGF-2 was increased, especially of IGF-2, whereas those of IGF-1 and growth hormone receptor decreased (Figure 4b). To determine whether these differences in mRNA expression were associated with circulating serum levels of IGF-1 and IGF-2, we measured these growth factors in serum. No significant differences were observed in serum IGF-1 or IGF-2 level between the WT and Ski mice with IGF-1 level (ng ml^{-1}) in WT being 147 ± 28 (mean ± s.e.m.) and in *c-Ski* mice 150 ± 37 ($n = 7$ per group), respectively, and IGF-2 level (ng ml^{-1}) in WT being 27.8 ± 1.5 and in *c-Ski* mice 34.6 ± 5.3 ($n = 7$ mice per group), respectively.

Ski *represses lipogenic gene expression*

Additional candidate-based expression profiling revealed that lipogenic gene expression was suppressed in *c-Ski* mice, relative to WT littermate mice (Figure 5). For example, qRT–PCR revealed that in the *c-Ski* mice, the greatest decrease in gene expression was in the mRNA encoding SREBP1c, the master lipogenic regulator. As insulin is a major activator of SREBP1c expression, we analysed mice in both fasted (low-insulin state) and non-fasted (higher insulin) states. This revealed that there was a greater-fold repression of SREBP1c expression in *c-Ski* mice in the non-fasted higher insulin state (Figure 5a) with a blunting of the Ski-dependent gene repression in the fasted state. Moreover, two major downstream targets of SREBP1c, *SCD-1* and *FAS* were both suppressed in the non-fasted state, whereas SCD-1, but not FAS, was significantly suppressed in the fasted state (Figure 5a). The expression of several NRs involved in the control of lipogenesis, lipid storage and metabolism was repressed in the *c-Ski* mice. These included the expression of the LXRα, PPARγ, GR and the orphan NR, RORα (Figure 5b).


a
Male
Female
% (mg/gBW)*100
0.75
0.50
0.25
0.00
WT TG
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EDL
Soleus
Gastroc
b
Male
Female
1.5
1.0
0.5
0.0
1.00
0.75
0.50
0.25
Inguinal
Epididymal
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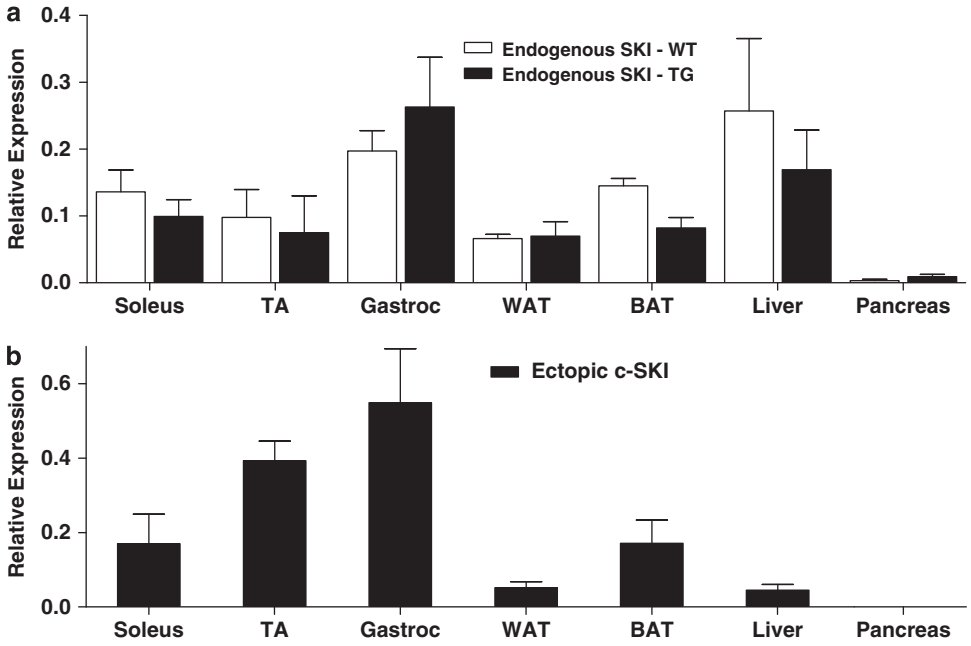

Figure 3 Ski expression in transgenic (TG) and wild-type (WT) animals. (**a**) Endogenous *m-Ski* mRNA expression and (**b**) Ectopic *c-Ski* transgene mRNA expression by quantitative RT–PCR in muscle and non-muscle tissues as shown in WT and TG male 15-week-old mice. TA, tibialis anterior; Gastroc, gastrocnemius; WAT, white adipose tissue; BAT, brown adipose tissue (Data are expressed as mean ± s.e.m., normalized to 18S with $n=3$ mice in each group).

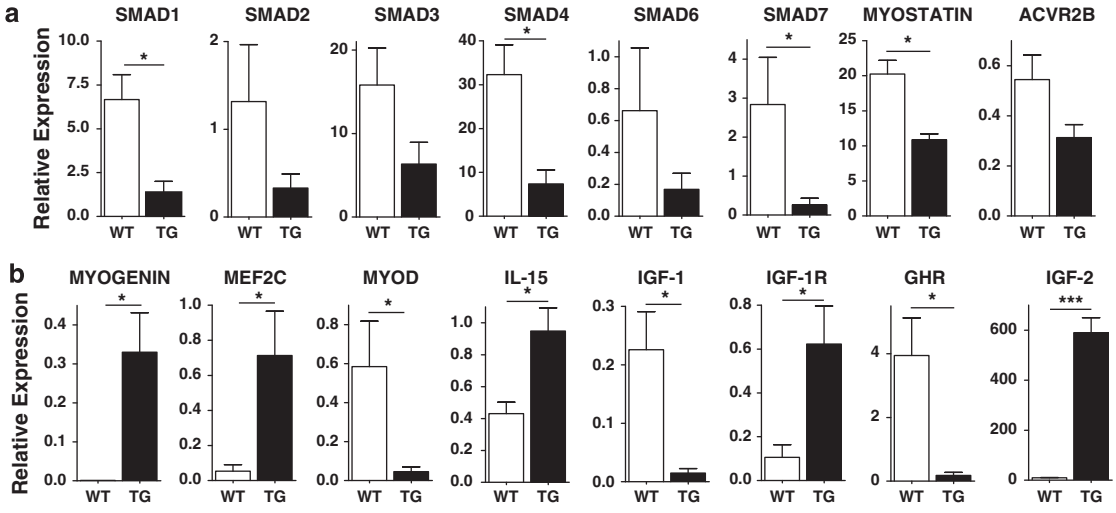

Figure 4 *c-Ski* mice have altered pattern of expression of key factors involved in skeletal myogenesis. Quantitative RT–PCR was used to assay expression of various transforming growth factor-β (TGF-β) and muscle-specific factors from skeletal muscle (gastrocnemius) in fasting 15-week-old male WT and TG mice. Data are expressed as mean ± s.e.m. relative to and normalized to 18S ($n=4$ mice in each group). (**a**) TGF-β family members including Smad1–4, 6 and 7 and myostatin. (**b**) Myogenic factors including myogenin, *MEF2c*, *MyoD* and IGF–GH signalling members. *$P<0.05$ and ***$P<0.0001$.

Figure 2 Skeletal muscle and fat pad masses in 15-week-old *c-Ski* mice. (**a**) Skeletal muscle weights in 15-week-old wild-type (WT) and transgenic (TG) mice after overnight fasting ($n=4$–7 mice in each group). All weights have been corrected for total body weight and are expressed as %. TA, tibialis anterior; EDL, extensor digitorum longus; SOL, soleus; Gastroc, gastrocnemius. *$P<0.001$ by two-way analysis of variance (ANOVA). (**b**) Fat pad weights in WT and TG mice. * all $P<0.01$ by two-way ANOVA. (**c**) Liver weight as a percent of body weight for male mice. (**d**) Haemoxylin and eosin (H & E)-stained sections of white adipose tissue (WAT) and brown adipose tissue (BAT) and liver. A representative sample from three WT and three TG mice is shown.

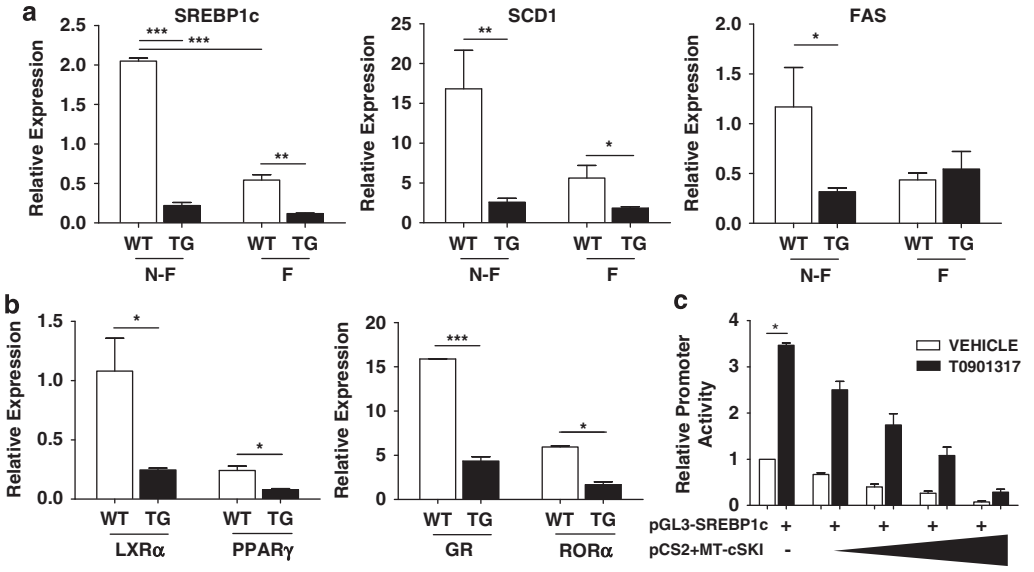

Figure 5 The expression of the lipogenic master regulator sterol-regulatory enhancer binding protein 1c (SREBP1c) and several related metabolic genes are markedly repressed in skeletal muscle of *c-Ski* mice. (**a**) *SREBP1c*, stearoyl-CoA desaturase-1 (*SCD-1*) and fatty acid synthase (*FAS*) mRNA skeletal muscle expression in non-fasting and fasting wild-type (WT) and transgenic (TG) mice. (**b**) Peroxisome proliferator-activated receptor-γ (*PPARγ*), liver X-receptor-α (LXRα) and retinoic acid receptor-related orphan receptor-α (*RORα*) mRNA gene expression in fasted WT and *c-Ski* mice (Data are expressed as mean ± s.e.m., normalized to 18S with $n=4$ in each group). *$P<0.05$, **$P<0.005$ and **$P<0.0001$. (**c**) Ski regulation of SREBP1c-promoter activity in C2C12 myogenic cells. Results are shown as relative luciferase activity to unliganded control without Ski transfection with vehicle (white columns) or LXR agonist T0901317 and are represented as mean ± s.e.m. of three independent experiments performed in triplicate. *$P<0.01$ as calculated by analysis of variance (ANOVA).

Interestingly, LXR and RORα have been described previously, as regulators of the *SREBP1c* promoter, in several cell lines derived from hepatic, adipose and muscle tissue and also *in vivo*.[26,34] In this context, LXR has also been reported to regulate the *SCD-1* and *FAS* promoters.[35,36] This suggests that Ski suppresses the expression of the two major regulators of lipogenesis, and this significantly affects the expression of the downstream target genes, *SCD-1* and *FAS*, which are involved in fatty acid biosynthesis.

Ski *represses the* SREBP1c *promoter*

The nuclear receptor LXR (in an agonist-dependent manner) and the orphan RORα have been demonstrated to transactivate the *SREBP1c* promoter in co-transfection experiments.[25,29] We thus examined whether co-transfected Ski transcriptionally regulated the activity of the *SREBP1c* promoter. Transfection analysis in C2C12 myogenic cells revealed that Ski repressed *SREBP1c* promoter activity in a dose-dependent manner (Figure 5c).[34] Interestingly, Ski co-transfection did not suppress LXR agonist-dependent induction. In concordance, mutation of the LXR-binding sites in the *SREBP1c* promoter did not affect Ski-mediated repression of the *SREBP1c* promoter (data not shown).

White adipose tissue from c-Ski *mice have unaltered metabolic gene expression*

To determine in white adipose tissue the pattern of gene expression in *c-Ski* versus WT mice, we performed a Taqman low density array experiment using a custom-made metabolic card with 96 genes known to be involved in metabolism (see Supplementary data). This analysis identified three possible candidate genes whose expression was potentially altered in *c-Ski* mice compared with WT mice. The increased expression in the *c-Ski* mice of cyclooxygenase 2 by 1.5-fold (log scale), myostatin by 2.2-fold and *MEF2c* by 3.64-fold, however, failed to reach statistical significance (see Supplementary data). In addition, no significant differences were observed in mRNA expression performed by qRT–PCR in the *c-Ski* mice compared with WT mice in these three transcripts and the nuclear receptors LXRα, GR or PPARγ (data not shown).

Skeletal muscle of c-Ski *mice display increased rates of fatty acid oxidation*

Gene expression profiling suggests that skeletal muscle from *c-Ski* mice has reduced lipogenic activity. To determine whether this is coupled to alterations in oxidative capacity, which may contribute to decreased adiposity, we performed palmitate oxidation experiments in skeletal muscle and measured the activity of several enzymes linked with oxidative metabolism, including cytochrome *c* oxidase, citrate synthase and hydroxyacyl-CoA dehyrogenase (Figure 6).[28] These studies were performed on quadricep skeletal muscle, which like the gastrocnemius muscle displayed decreased expression of the lipogenic regulator, SREBP1c, in *c-Ski* mice relative to WT mice (data not shown).

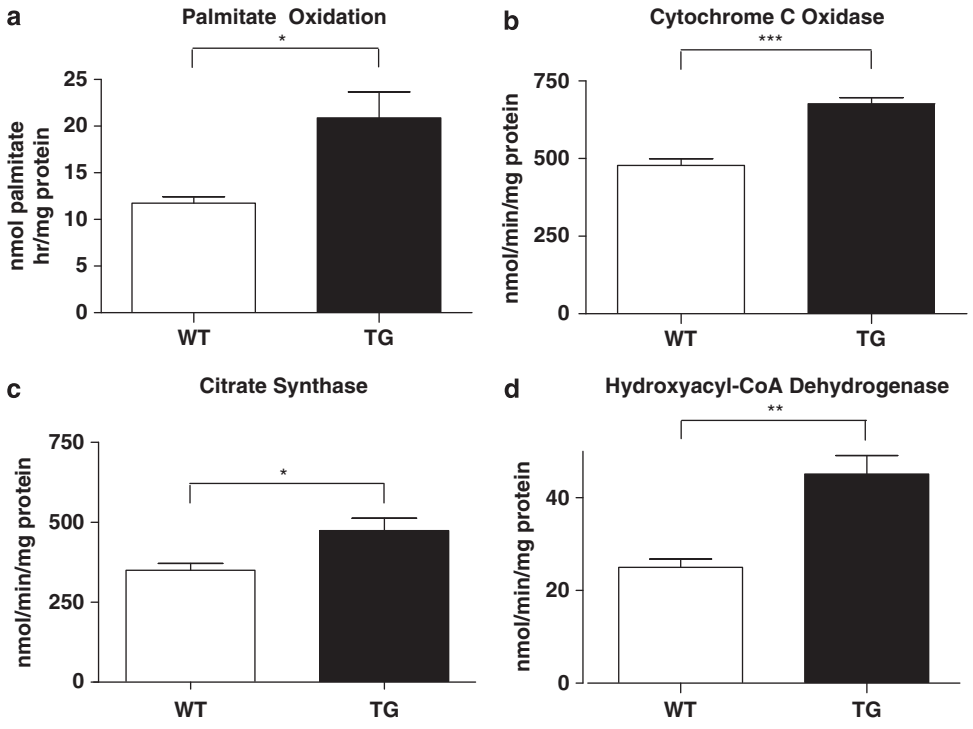

Figure 6 Skeletal muscle of *c-Ski* mice has increased fatty oxidative capacity. (**a**) Palmitate oxidation rate in skeletal muscle homogenates (nmol palmitate h per mg protein) and (**b**) Cytochrome *c* oxidase, (**c**) citrate synthase and (**d**) hydroxyacyl-CoA dehydrogenase enzymatic activities (nmol min^{-1} per mg protein) in skeletal muscle homogenates from WT and *c-Ski* mice ($N = 5$–6 mice). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.0001$.

We found that skeletal muscle of *c-Ski* mice had significantly increased palmitate oxidation and significantly elevated activity of oxidative enzymes compared with WT mice. In the *c-Ski* mice, an increased enzyme activity was observed for cytochrome *c* oxidase, citrate synthase and hydroxyacyl-CoA dehydrogenase.

Circulating lipids, insulin, adiponectin and glucose tolerance in c-Ski *mice*

Consistent with the lean phenotype of the *c-Ski* mice, we observed significantly decreased serum levels of leptin and adiponectin, whereas no significant differences in fasting insulin, non-esterified fatty acid or triglyceride levels were observed (Figure 7a). To investigate the effects of the lean phenotype on glucose tolerance, we performed glucose tolerance tests on Ski and WT littermate controls. Interestingly, glucose tolerance in *c-Ski* mice was mildly impaired compared with WT littermate controls ($P < 0.05$; Figure 7b).

Discussion

The paramount role of the *Ski* gene in myogenesis and TGF-β signalling has been well-characterized *in vitro* and *in vivo*.[2,9] Although, the original description of the *c-Ski* transgenic mouse model showed increased muscle mass and reduced adiposity in these mice, the underlying mechanism for this latter effect has remained obscure. In this study, candidate-based expression profiling demonstrates that the skeletal muscle of *c-Ski* transgenic mice display aberrant expression of several critical transcriptional cascades that control myogenic and lipogenic signalling pathways that influence body composition. For example, Ski expression leads to: (i) attenuation of *Smad* gene expression; (ii) suppression of myostatin expression; (iii) increased myogenin, *MEF2c*, insulin-like growth factor 1 receptor and *IGF-2* expression; and (iv) decreased expression of LXRα, PPARγ, RORα and *SREBP1c* (and the downstream lipogenic target genes). These nuclear hormone receptors and SREBP1c are hierarchical regulators of fatty acid biosynthesis and storage.

In keeping with Ski's role in suppressing Smad-dependent TGF-β signalling, *c-Ski* mice had decreased expression of *Smad1*, *4* and *7* and myostatin expression. Myostatin, a TGF-β superfamily member, operates in an opposing manner in skeletal muscle and adipose tissue. For example, myostatin is a negative regulator of skeletal muscle mass and it induces adiposity by promoting adipogenesis.[12,37,38] Myostatin-deficient mice and humans have increased muscle mass and decreased adipose tissue mass.[37] Ski suppression of myostatin and GR expression, a transcriptional activator of the myostatin promoter,[39] is therefore consistent with the lean phenotype of the mice. The altered expression of myostatin in the *c-Ski* mice in association with increased myogenin and *MEF2c* expression, though unexpected (compensatory)

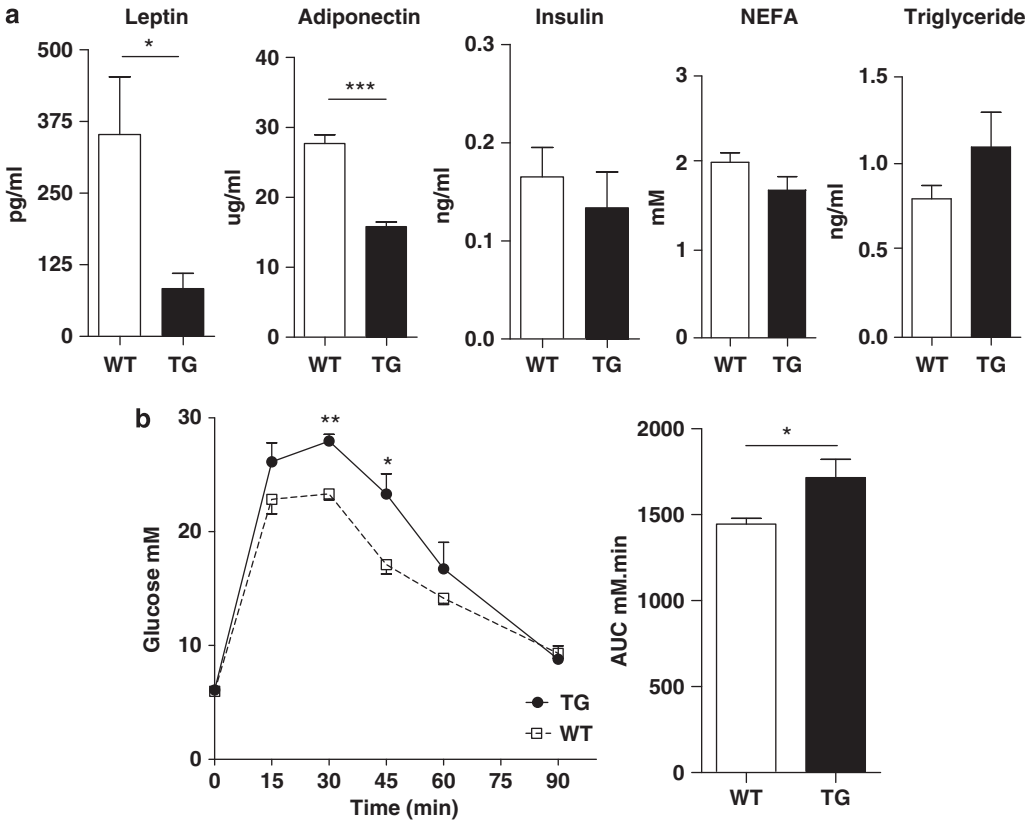

Figure 7 Serum adipokines and lipids and glucose tolerance in *c-Ski* mice. (**a**) Serum levels of leptin, adiponectin, insulin, non-esterified fatty acids (NEFAs) and triglycerides from overnight fasted 15-week-old male wild-type (WT) and *c-Ski* mice ($n=7$–9 mice per group) $^{*}P<0.05$, and $^{***}P=0.0001$. (**b**) Glucose tolerance testing of Ski ($n=3$) relative to 15–week-old WT littermate controls ($n=5$). $^{**}P=0.0001$ and $^{*}P<0.05$ at 30 and 45 min, respectively and glucose tolerance results expressed as area under the curve (AUC) with $^{*}P<0.05$.

repression of *MyoD* suggests that Ski targets several myogenic differentiation genes.

The alteration of genes involved in the GH–IGF system also implicates a role for Ski in growth factor regulation of both skeletal muscle and adipose mass. We observed a marked increase in *IGF-2* and insulin-like growth factor 1 receptor mRNA levels, but a compensatory decrease in *IGF-1* and *GHR* mRNA levels.[40] In particular, *IGF-2* expression was markedly elevated in Ski skeletal muscle. Both IGF-1 and IGF-2 are critically involved in skeletal muscle development, with IGF-2 autocrine actions being essential for the differentiation of satellite cells.[31] Amino acid and mammalian target of rapamycin (mTOR) signalling has been shown to control IGF-2 transcription in skeletal muscle cells,[33] which acts through the insulin-like growth factor 1 receptor to alter phosphoinositide 3-kinase (PI3K)–Akt pathway and, therefore, promotes myogenesis and decreases adipogenesis.[32]

Interestingly, we observed decreased expression of several nuclear hormone receptors that have been implicated in a 'fatty' phenotype, including LXRα, PPARγ, RORα and the GR. Furthermore, the mRNA encoding *SREBP1c* (a master regulator of lipogenesis) and its downstream fatty acid biosynthetic target genes, *FAS* and *SCD-1* were decreased in Ski mice. *SREBP1c* is an important LXR and ROR target gene, expression of which is elevated in skeletal muscle from morbidly obese patients, and decreases with weight loss in conjunction with reductions in intramyocellular lipid and improvements in insulin sensitivity.[41] In this context, it is interesting to note that mouse models with attenuated LXRα and RORα expression display reduced adiposity and resistance to diet-induced obesity.[26] Overall, the NR expression footprint observed in the *c-Ski* mice is concordant with reduced lipogenesis that presumably contributes, in part, to the lean phenotype of these animals.

The reduced adiposity in *c-Ski* mice is also associated with increased skeletal muscle fatty acid oxidative capacity. Several other genetically manipulated mouse models have a lean phenotype that seems to be driven, in part, by elevated fat oxidation in skeletal muscle.[42–44] Of these lean mice, perhaps the most relevant for the *c-Ski* mice are animals deficient in SCD-1, as we observed a reduced expression of *SCD-1* in muscle from the *c-Ski* mice. Stearoyl-CoA desaturase-1 expression has been shown to correlate negatively with fatty acid oxidation in skeletal muscle from

humans,[45] mice[42] and in L6 myotubes.[46] Our finding that Ski represses the expression of lipogenic genes, such as *SCD1*, therefore, provides a potential explanation for the increased fatty acid oxidation observed in skeletal muscle of the *c-Ski* mice. Alternatively, alterations of physical activity or thermogenesis may explain the reduced adiposity of the *c-Ski* mice. An analysis of these other potential contributing factors to the lean phenotype of the *c-Ski* mice is the focus of current studies.

The finding of mild glucose intolerance in *c-Ski* mice compared with WT littermates controls was surprising, but may be consistent with our observation in the *c-Ski* mice of a reduction in PPARγ expression in skeletal muscle and reduced adiposity, and also decreased adiponectin levels observed in the *c-Ski* mice.[47,48] Interestingly, knockout of the *PPARγ* gene in skeletal muscle leads to severe glucose intolerance.[49] In addition, these PPARγ-deficient mice have progressive severe insulin resistance with aging.[49] In contrast to the *c-Ski* mouse the PPARγ mice have increased body fat as evidenced by heavier epididymal fat pads than their WT littermate controls.[49] Presumably as the *c-Ski* mice still do have detectable levels of PPARγ expression though an overall repressed lipogenic gene profile (including decreased levels of other NRs, such as GR, LXRα and RORα), the degree of glucose metabolism is not severely impaired at least on a normal chow diet. It will be thus interesting to determine in future studies whether the *c-Ski* mice are resistant to diet-induced obesity and whether a high-fat diet may alter glucose tolerance and insulin sensitivity.

The overall depots of both white and brown adipose tissues were significantly decreased in *c-Ski* mice, raising the possibility that adipose gene expression may be altered in the *c-Ski* mice. Our examination of gene expression changes in white adipose tissue failed to show any significant changes in the 96 genes we studied in the *c-Ski* mice. Interestingly, recent evidence suggests that brown adipose tissue and skeletal muscle are derived from the same cell lineage from myogenic factor-5 (Myf-5)-expressing cells.[50] Though our results suggest Ski alters adipose tissue development through effects on skeletal muscle gene expression and metabolism, further studies will be required to determine whether Ski directly regulates white and brown adipocyte differentiation.

In summary, in these new studies we have shown that the proto-oncogene *Ski* targets the master lipogenic regulator *SREBP1c* and several NRs involved in adipogenesis and metabolism in skeletal muscle. These results and those we present regarding Ski's effects on skeletal muscle metabolism and energy expenditure suggest that Ski may have a major role in body composition development and hence risk for obesity and metabolic disease.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on International Journal of Obesity website (http://www.nature.com/ijo)