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Functional characterization of a promoter polymorphism that drives ACSL5 gene expression in skeletal muscle and associates with diet-induced weight loss

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Abstract

Diet-induced weight loss is affected by a wide range of factors, including genetic variation. Identifying functional polymorphisms will help to elucidate mechanisms that account for variation in dietary metabolism. Previously, we reported a strong association between a common single nucleotide polymorphism (SNP) rs2419621 (C>T) in the promoter of acyl-CoA synthetase long chain 5 (ACSL5), rapid weight loss in obese Caucasian females, and elevated ACSL5 mRNA levels in skeletal muscle biopsies. Here, we showed by electrophoretic mobility shift assay (EMSA) that the T allele creates a functional cis-regulatory E-box element (CANNTG) that is recognized by the myogenic regulatory factor MyoD. The T allele promoted MyoD-dependent activation of a 1089-base pair ACSL5 promoter fragment in nonmuscle CV1 cells. Differentiation of skeletal myoblasts significantly elevated expression of the ACSL5 promoter. The T allele sustained promoter activity 48 h after differentiation, whereas the C allele showed a significant decline. These results reveal a mechanism for elevated transcription of ACSL5 in skeletal muscle of carriers of the rs2419621(T) allele, associated with more rapid diet-induced weight loss. Natural selection favoring promoter polymorphisms that reduced expression of catabolic genes in skeletal muscle likely accounts for the resistance of obese individuals to dietary intervention.

Keywords

fatty acid; genetics; MyoD; obesity

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Obesity is a growing global health pandemic. According to the World Health Organization (WHO), the number of overweight (BMI≥25) adults will exceed 2 billion in 2015. This condition poses a global health issue, since the risk of many chronic diseases, including cerebrovascular and cardiovascular diseases (1), type II diabetes (2), musculoskeletal disorders (3), and cancers (4), increases because of obesity. Although exercise and food intake modulate caloric homeostasis, differences in the response to diet-induced weight loss depend on interindividual ability to store and expend energy.

Skeletal muscle makes up 40 –50% of the body mass in an adult and utilizes free fatty acids (FFAs) as a source of energy. Generally, fat is stored as triglycerides in adipose tissues and becomes mobilized to the sites of utilization in the form of FFA. The uptake of FFA by skeletal muscle for metabolism is initiated by transmembrane acyl-CoA synthetase long-chain (ACSL) proteins that esterify FFAs to acyl-coenzyme A (acyl-CoA) molecules. Acyl-CoA species are used mainly in both the synthesis of cellular lipids and the degradation of fatty acids *via* β-oxidation. Six members of ACSL family genes have been identified in humans and rodents. In humans, ACSL5 transcripts are distributed in a wide range of tissues, and a high expression has been reported in uterus and spleen (5). In rats, ACSL5 mRNA is abundant in liver, brown adipose tissue, and duodenal mucosa (6) and less abundant in other tissues, including skeletal muscle (7). ACSL5 overexpression in liver cell lines promotes fatty acid uptake, which results either in increased storage (8) or in increased β-oxidation (9). Thus, small increases in the expression of ACSL5 in skeletal muscle could have profound effects on FFA utilization.

Previously, we reported a strong association between the common single nucleotide polymorphism (SNP) rs2419621 and rapid weight loss in obese Caucasian females in response to restricted diet (7). The SNP located 12 nucleotides upstream of the second transcription start site of the ACSL5 gene (Fig. 1*A*) is characterized by a cytosine [rs2419621(C)] to thymine [rs2419621(T)] transition (Fig. 1*B*). We also showed that the T allele is associated with a 2.2-fold increase of ACSL5 transcript level in skeletal muscle biopsies when compared to noncarriers (7). Here, we tested whether the T allele is functionally responsible for the up-regulation of ACSL5 expression in skeletal muscle.

MATERIALS AND METHODS

Construction of vectors

Human ACSL5 promoter was amplified by PCR with the primers 5′-GAA *GAG CTC* GCA AAT GAC AAG TGC TCC TCC AGC C-3′ (sense primer; *Sac*I) and 5′-GAA *AGA TCT* GAG CGT CCA TGC AGG CAG AAG GC-3′ (antisense primer; *Bgl*II) and was then subcloned into a TOPO vector (Invitrogen Inc., Carlsbad, CA, USA). The amplicon was released by *Sac*I-*Bgl*II double digestion and subcloned into pGL3-basic vector (Promega, Madison, WI, USA) to create pGL3-ACSL5 plasmids. The T-allele was introduced in the pGL3-ACSL5 plasmid using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) based on the manufacturer's instructions. Sequences of the sense primer (SP) and antisense primer (AP) (Operon Biotechnologies, Inc., Huntsville, AL, USA) are 5′-CAA GTG CTC CTC CAG CTG TGA GAT GAG CCA GAG G-3′ and 5′-CCT CTG GCT CAT CTC ACA GCT GGA GGA GCA CTT G-3′, respectively. The CMV-promoter based

pXJ40-MyoD expression vector and the mouse myogenin-luciferase reporter were made previously (10). For the construction of pGEX-5X-1-MyoD, the 1.7 kb *Mus musculus* MyoD cDNA was released from the pACT-MyoD plasmid in the Matchmaker™ mammalian two-hybrid system (Promega, Fitchburg, WI, USA) by *Bam*HI digestion and was then subcloned into a *Bam*HI-digested pGEX-5X-1 vector (a gift of Dr. Heidi McBride, University of Ottawa Heart Institute, Ottawa, ON, Canada). The sequence of all plasmid constructs was checked by sequencing (Applied Biosystems, Foster City, CA, USA).

Fusion protein purification

DH5α competent cells transformed with pGEX-5X-1-MyoD were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C to generate glutathione S-transferase (GST)-MyoD fusion proteins. At 3 h postinduction, the cells were sonicated, and supernatants were collected. Fusion proteins were purified by absorption to glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) and were eluted with 50 mM Tris (pH 8.0) and 20 mM reduced glutathione (Sigma-Aldrich). GST-MyoD fusion proteins were detected on a SDS-PAGE gel, followed by Coomassie-brilliant blue staining for verification.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (11), using double-stranded oligonucleotides that carry the C allele 5′-AAA CTA AGC AAA TGA CAA GTG CTC CTC CAG CCG TGA GAT GAG CCA GAG GAT GGA A-3′ or the T allele 5′-AAA CTA AGC AAA TGA CAA GTG CTC CTC CAG CTG TGA GAT GAG CCA GAG GAT GGA A-3′ (rs2419621 SNP is underscored). The purified GST-MyoD fusion protein (2 μg) from bacteria was used in EMSA. Monoclonal MyoD antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against mouse MyoD protein was added for a supershift reaction. Intensity of shifted complexes was quantified on a phosphor storage screen (Amersham, Piscataway, NJ, USA).

Cell culture and transient transfections

CV1 and C_2C_{12} cells were maintained in 1× minimum essential medium (MEM; Mediatech, Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were allowed to grow until 80% confluence in 10-ml plates in a humidified incubator of 37°C and 5% CO_2. One day before transfection, cells were seeded into 6-well tissue culture plates (Corning Inc., Corning, NY, USA). Lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturer's instruction. Briefly, lipoplexes containing 0.1 μg of expression vector, 0.7 μg of reporter plasmid, and 6 μl of lipofectamine 2000 were added to wells of 80 to 90% confluent cells. After 6 h, cells were washed with 1× phosphate-buffered saline (PBS; 80 g NaCl/L, 2 g KCl/L, 14.4 g Na_2HPO_4/L, and 2.4 g KH_2PO_4/L, pH 7.4) and were incubated in 20% FBS-containing media. Differentiation in C_2C_{12} cells was initiated by adding 3% horse serum (Sigma-Aldrich) in serum-free MEM, followed by 24 and 48 h of incubation.

Luciferase and β-galactosidase assays

Luciferase assays were performed as described previously (10). β-Galactosidase assays were performed according to the manufacturer's instructions (Promega) with modifications.

Briefly, 50 μl cell lysate and 20 μl ice-cold β-gal phosphate buffer (60 mM Na_2HPO_4, 45.8 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, and 1.0 mM $MgSO_4{\cdot}7H_2O$) were mixed in a 96-well plate and incubated at 37°C for 10 min. To each sample, 50 μl ONPG buffer (200 mM Na_2HPO_4, pH 7.3; 2 mM $MgCl_2$; 100 mM β-mercaptoethanol; and 1.33 mg/ml ONPG) was added and incubated to 37°C until yellow color developed. β-Galactosidase activity was determined on a microplate spectrophotometer (BioTek, Winooski, VT, USA). Luciferase activities were normalized to activities in cells transfected with wild-type pGL3-ACSL5 plasmids. Mean fold activities of the rs2419621 (T allele) promoter response to mouse MyoD was considered different from the rs2419621 (C allele) at the $P < 0.05$ level by independent sample *t* test. Luciferase activities were assessed on a luminometer (Montreal-Biotech Inc., Montreal, QC, Canada) following the manufacturer's instructions.

Immunocytochemistry

C_2C_{12} cells were cultured in two-chambered glass plates that were first coated with 4% gelatin. Next, cells were fixed with ice-cold methanol at −20°C for 10 min and were permeablized with 0.1% Triton-X 100 for 10 min, followed by 1 h of blocking with 5% FBS in 1× PBS. Mouse monoclonal MF-20 antibody (Cornell University Medical College, Cornell, NY, USA) against myosin heavy chain was hybridized to the cells for 1 h, and Alexa 488 (Invitrogen) against mouse antibodies was incubated with the cells. DAPI was used for nuclei counterstaining.

RESULTS

rs2419621(T) allele creates a putative E box

Consultation of HapMap identified 3 SNPs in linkage disequilibrium with rs2419621 (rs17129754, rs11195951, and rs2419627), but these polymorphisms lie within introns of ACSL5. Thus, barring the existence of other unknown functional SNPs in the promoter, the T allele was considered a good functional candidate because it creates a *de novo* consensus E-box (CANNTG) cis-regulatory element (Fig. 1), a putative binding site for basic helix-loop-helix myogenic regulatory factors (MRFs) and E proteins (12–15). During skeletal muscle differentiation, MRFs and E proteins form transcriptional complexes that control the expression of many downstream genes, including MEF2C (16), skeletal α-actin (17), and muscle creatine kinase (18). To test whether the existence of this *de novo* E box can be recognized by MRFs, ^{32}P-labeled double-stranded DNA probes carrying either the T or C allele were incubated with GST-MyoD chimeric proteins in an EMSA. MyoD interacted with the rs2419621(T) probe (Fig. 2, lane 8) more strongly than the rs2419621(C) probe (Fig. 2, lane 3). Supershift with antibody to MyoD confirmed its presence in the shifted complex (Fig. 2, lanes 5 and 10). Thus, the two preexisting E-box elements are recognized by MyoD (Fig. 2, lanes 3 and 6) *in vitro,* and the third E box created by rs2419621(T) recruits additional MyoD to the ACSL5 promoter.

The rs2419621(T/C) ACSL5 promoter alleles are differentially activated by MyoD and during muscle differentiation

We asked whether MyoD binding to this additional E-box site enhances ACSL5 expression, and if so, by what degree. CV1 cells (African green monkey kidney; MRF-free) were

cotransfected with a CMV enhancer-driven MyoD expression vector and ACSL5-luciferase reporter vectors (carrying either the rs2419621 T or C allele) or a mouse myogenin reporter vector. All luciferase activities were expressed related to the rs2419621(C)-allele ACSL5 reporter. MyoD activated the myogenin promoter 3-fold, the rs2419621(T)-allele luciferase promoter 2-fold, and the rs2419621(C) reporter 1.5-fold when compared to empty expression vector in CV1 cells (Fig. 3). This result is consistent with the EMSA results and suggests that the third E-box element recruits more MyoD to the promoter.

Next, we asked whether the ACSL5 rs2419621(T or C)-luciferase reporter constructs are differentially regulated in response to muscle differentiation when MRF expression is up-regulated. Prior to muscle differentiation, the rs2419621 polymorphism did not appear to affect the activity of ACSL5 promoter (Fig. 4). A progressive difference in the activity of ACSL5 promoter was noted during the course of muscle differentiation between the two sequence variants. The activity of ACSL5 promoter of the rs2419621(C) allele increased then declined with muscle differentiation, whereas the activity of the rs2419621(T) allele remained elevated. This result is consistent with our previously reported finding of elevated ACSL5 mRNA expression in patients with the rs2419621(T) variant (7).

DISCUSSION

Our study demonstrates that the rs2419621(T) allele forms a functional E-box element upstream of the second ACSL5 isoform transcript start site. MyoD physically and functionally interacts with this *de novo* E box to up-regulate ACSL5 expression in muscle cells. The reference rs2419621(C) allele lacks the third E-box element and is associated with a lower ACSL5 promoter activity in differentiating skeletal muscle. The two E-box elements make the ACSL5 promoter minimally responsive to MyoD, consistent with early work from Weintraub *et al.* (19) that showed two or more E boxes are required for a promoter to be MyoD responsive. This is the first example of a MyoD binding polymorphism conferring differential promoter activity of a metabolic gene.

The regulation of ACSL isoforms is largely unknown. The present findings represent a new molecular mechanism by which one ACSL isoform, ACSL5, is differentially regulated at the transcriptional level. Adenoviral-mediated overexpression of ACSL5 in liver cell lines produced apparently contradictory results. Mashek *et al.* (8) showed in rat hepatoma cells that ACSL5 promotes fatty acid uptake destined for TG synthesis and storage, while recently Zhou *et al.* (9) showed in human HepG2 cells that it results in an increase of nearly 50% in β-oxidation associated with a trend toward TG reduction. In rat liver, ACSL5 mRNA increased after refeeding following food deprivation and paralleled increase in β-oxidation (8).

Our previous results in humans where carriers of the rs2419621(T) allele display higher levels of skeletal muscle ACSL5 mRNA and are better responders to diet (7) are consistent with the finding of Zhou *et al.* (9). Moreover, carriers of the rs2419621(T) allele have lower total cholesterol and tend to have lower low-density lipoproteins and triglycerides (7). Adenoviral-mediated overexpression of ACSL5 in rat hepatoma cells leads to Ad-ACSL5 expression in both mitochondria and endoplasmic reticulum (6). However, because of its

location 12 bp upstream of the second major transcription start site (Fig. 1), rs2419621 SNP is likely to influence primarily transcription from this second transcription start site. It is conceivable that transcripts from this second major transcription start site are directed mainly to the mitochondria and thus involved in fatty acid oxidation. Transcripts from the two other transcription start sites might be directed to other cellular compartments, including endoplasmic reticulum, and thus involved in lipid synthesis and storage. Taken together, these data suggest that the rs2419621 SNP influences the rate of weight loss by increasing ACSL5 levels and promoting β-oxidation over TG synthesis and storage.

Ideally, replication of the association of rs2419621 with diet-induced weight loss in other cohorts will be necessary to confirm whether it predicts dietary response. It is worth noting that rs2419621 has not been associated with obesity in genome-wide association studies, perhaps because it is not represented on commercial arrays. However, genes that predispose to weight gain may not necessarily impair the ability to lose weight. For example, several recent studies saw no association of the well-known FTO gene with diet-induced weight loss (20, 21) or with energy expenditure (22). Similarly, the rs2419621 (T) allele in ACSL5 might not associate with weight gain, but predict weight loss.

The CC genotype has >50% prevalence in each of four populations (67.2% in 116 CEU, 51.1% in 90 HCB, 71.4% in 84 JPT, and 71.7% in 120 YRI) sampled in the International HapMap project. Natural selection may have favored carriers of the C allele, enabling them to survive famine by promoting fatty acid storage over catabolism. Promoter polymorphisms that affect expression of metabolic genes in skeletal muscle may constitute a general mechanism underlying variation in the response to diet-induced weight loss.

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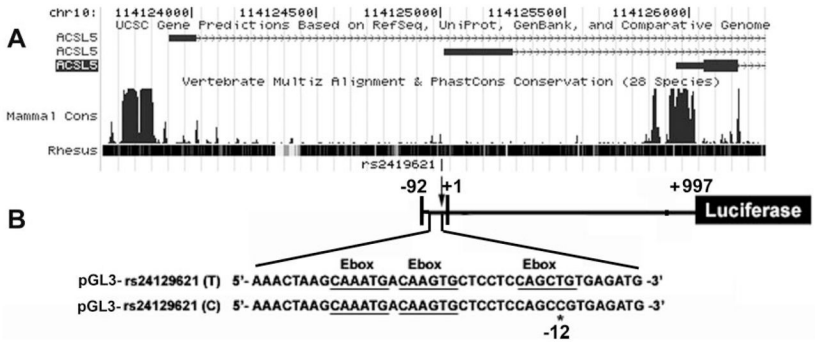

Figure 1.

The human ACSL5 promoters. *A*) Diagram of the three human ACSL5 promoters and the location of the rs2419621 SNP, −12 bp from the start of the second ACSL5 promoter. *B*) Sequence of the proximal region near the second promoter carries either two E-box elements or a third E box created by the rs2419621(T) allele (asterisk). The 1089-bp promoter fragment extending from −92 bp of the second start site to +997 bp was cloned into the pGL3 luciferase vector.

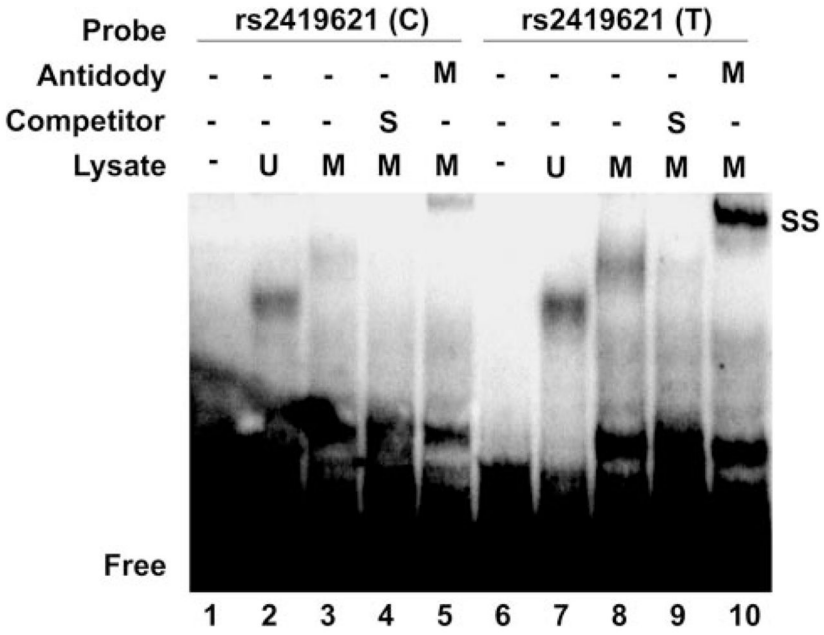

Figure 2.

The ACSL5 rs2419621(T) allele is a functional MyoD binding site. Gel mobility shift assays with bacterially expressed GST-MyoD reveals a weak interaction with the rs2419621(C) major allele (lane 3) but a strong interaction with the rs2419621 (T) allele (lane 8). Supershift (SS) with a MyoD antibody confirmed the presence of MyoD in the shifted complex (lanes 5 and 10). Unprogrammed bacterial lysate produces a shift unrelated to MyoD (lanes 2 and 7). The oligonucleotides are shown in Fig. 1*B*. The C-allele oligonucleotide contains 2 E-box elements, whereas the T-allele oligonucleotide contains 3 E-box elements. Gel shift with a single E box corresponding to the T allele was not sufficient to produce a gel shift (data not shown) due to the cooperative nature of MyoD binding.

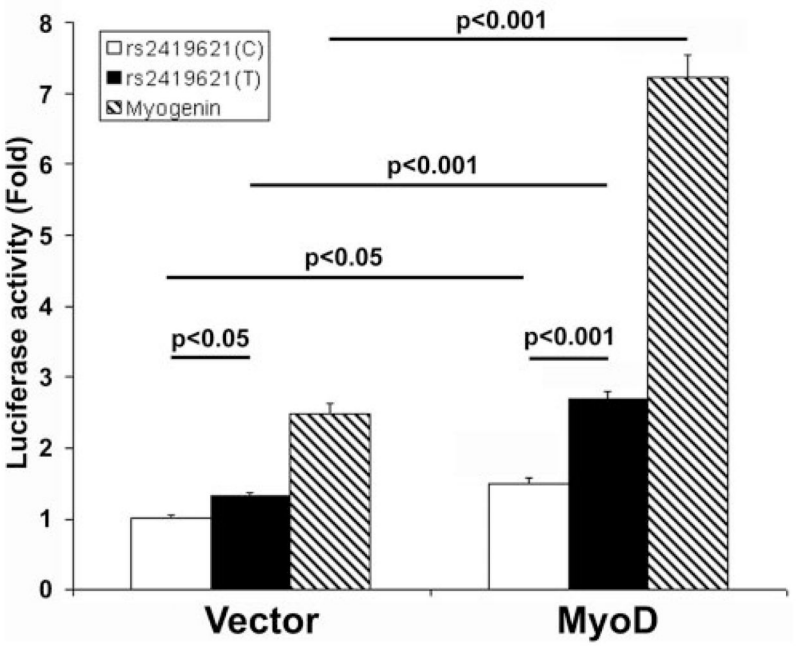

Figure 3.

MyoD differentially activates the ACSL5 promoter according to the rs2419621 genotype. The ACSL5 promoter luciferase construct bearing the C allele was weakly activated by MyoD, whereas the promoter bearing the T allele of rs2419621 was markedly activated by MyoD in transiently transfected CV1 cells. The MyoD-dependent mouse myogenin promoter served as a positive control. Luciferase activities were compared in the absence (vector) or presence of MyoD. $n = 5$ experiments.

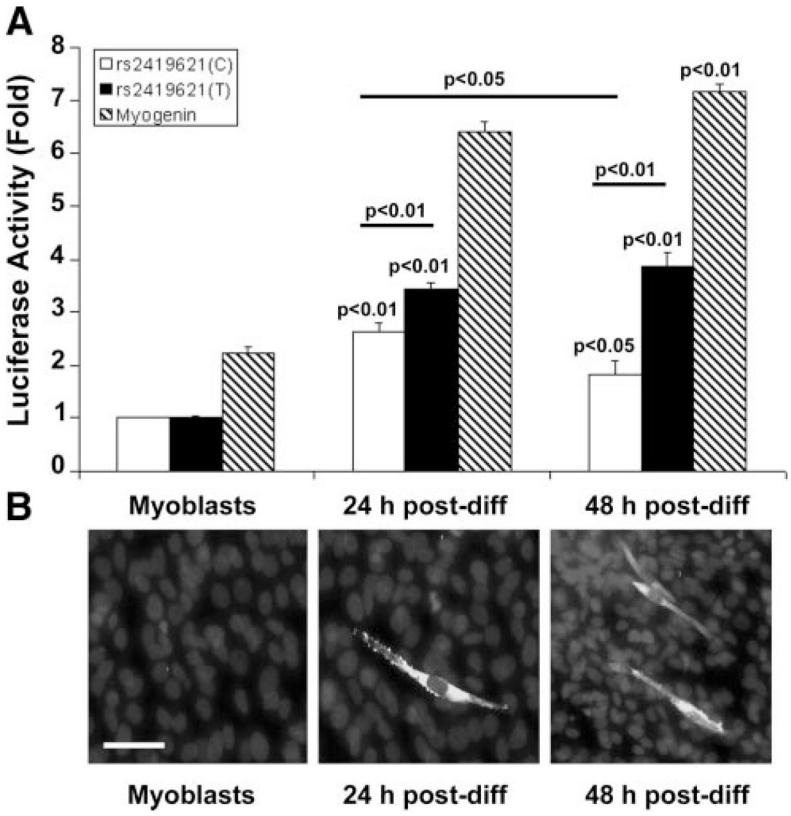

Figure 4.

Muscle differentiation activates the ACSL5 promoter. *A*) C_2C_{12} myogenic cells were transiently transfected with ASCL5 promoter-luciferase constructs and induced to differentiate by serum withdrawal. The rs2419621(C) allele bearing ACSL5 promoter is activated 24 h after muscle differentiation, but expression declines by 48 h. In contrast, the activity of the promoter bearing rs2419621(T) allele remains elevated. Expressed luciferase activity is standardized to the rs2419621(C) allele in undifferentiated myoblasts. The myogenin promoter served as a positive control. $n = 5$ experiments. *B*) Myogenic differentiation was monitored by myosin heavy chain immunofluorescence. Nuclei were stained with DAPI. Scale bar = 50 μm.