Effects of fenofibrate on Semaphorin 6B gene expression in rat skeletal muscle

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Abstract. Semaphorin 6B is a member of the semaphorin family of signaling proteins, which is implicated in a variety of biological processes, such as axon guidance and muscle regeneration. PPARα is known to be a regulator of semaphorin 6B in human cancer cell lines. In this study, we examined the effect of fenofibrate as a PPARα activator on the expression of the Sema6B gene in rat skeletal muscle. A total of 18 rats were divided into two groups: one fed with standard chow (control group), and the other with fenofibrate chow (treatment group) for 4 weeks by gavage with 30 mg/kg per day. The expression of rSema6B mRNA was analyzed by qPCR. rSema6B protein content in rat skeletal muscle was measured by Western blotting. A significant down-regulation of rSema6B was observed at both the mRNA and protein levels. Using the ChIP assay, a putative PPARα binding region (PPRE) was identified in the rSema6B promoter. We conclude that the expression of Sema6B is down-regulated by fenofibarte in rat skeletal muscle.

Introduction

Restoring skeletal muscle function and physiological properties after injury depends on regenerative mechanisms that coordinate the remodeling of the intramuscular motoneuron network and neuromuscular connections (1,2). Such mechanisms remain unclear, although both attractive and repulsive axon guidance molecules belonging to several families of proteins, such as semaphorins (3-5), netrins (6), ephrins (7) and slits (8,9), may be involved in the signaling pathway.

Semaphorins are members of a large highly conserved family of signaling molecules. These were initially identified as playing a role in axon guidance and neural network forma-

tion (10,11), and later recognized to control diverse cellular processes ranging from cell migration and immune response to angiogenesis and cancerogenesis (12). To date, more than 20 semaphorins have been identified and divided into 8 classes. Classes 1-2 are found in invertebrates, classes 3-7 are vertebrate semaphorins, while class 8 contains viral semaphorins (13). Semaphorins are secreted membrane-anchored or transmembrane proteins characterized by a conserved semaphorin domain of about 500 amino acids. In contrast to the other semaphorins, class 6 semaphorins do not contain additional structural motifs such as immunoglobulin-like domains (classes 2-4 and 7), thrombospondin repeats (class 5) and basic domains (class 3) (5). However, their cytoplasmic domains contain several proline-rich putative SH3 domain binding sites and interact with several signaling proteins, such as Abl (14) and enabled/vasodilator-stimulated phosphoprotein-like protein (14,15). In rat embryos, Sema6B (semaZ) mRNA expression was shown to vary spatio-temporally during brain development (16).

During embryonic development, mouse Sema6B mRNA is expressed in subregions of the nervous system and is particularly prominent in muscles (17). Furthermore, Sema6B is highly expressed in some human tumor cell lines, and its expression is down-regulated by all-trans retinoic acid, probably via heterodimerization with peroxisome proliferator activated receptors (PPARs), which bind to a response element (PPRE) on the human Sema6B gene (18-20).

PPARs are transcription factors belonging to the superfamily of nuclear receptors (21). They are encoded by three genes located on different human chromosomes: PPARα, PPARβ/δ and PPARγ. PPARα is expressed in the liver as well as in skeletal muscle (22), where it plays a key role in the control of lipid metabolism and homeostasis (23). PPARα agonists of the fibrate class, such as fenofibrate, clofibrate, and gemfibrozil, are used clinically to control dyslipidemia primarily by transcriptionally regulating genes involved in both peroxisomal and mitochondrial fatty acid β-oxidation and glucose metabolism (24,25). Although the target-driven hepatic effects of PPARα treatment have been extensively studied, such responses in skeletal muscle are not as well understood. Lipid-lowering drugs such as fibrates have been implicated in the occurrence of myalgias (26-28) and rhabdomyolysis, in particular when co-administered with other drugs (29,30).

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In the present study, we showed that the Sema6B gene is down-regulated in rat skeletal muscle by fenofibrate (FF) treatment. Furthermore, we demonstrated using the ChIP assay that PPARα binds to a putative PPRE in the Sema6B promoter in rat skeletal muscle, suggesting that Sema6B down-regulation is mediated by PPARα.

Materials and methods

Chemicals. Fenofibrate and methylcellulose (400 centipoise) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primers for RT-PCR, qPCR and ChIP were synthe-sized at the Atomic Energy Commission of Syria (AECS). dNTPs, reverse transcriptase, Taq polymerase and RNase were purchased from Invitrogen (Carlsbad CA, USA). The cocktail of protease inhibitors (complete) and the BM chemi-luminescence kit were purchased from Roche (Mannheim, Germany). Antibodies directed against PPARα and β-actin were purchased from Santa Cruz (CA, USA). All other prod-ucts were of reagent grade.

Animals. Male and female Wistar rats, 7-8 weeks of age and weighting 150-170 g, were obtained from the AECS animal house post-ablactation. Animals were housed in rodent cages with 5-7 rats per cage and acclimatized for 1 week with a 12-h light/dark cycle in an air-conditioned animal room at 22˚C. The experiment was carried out on 18 animals divided into two groups: 9 rats treated with 300 mg/kg of the PPARα-selective agonist FF dissolved/suspended in 0.25% methylcellulose (treatment group), and 9 rats similarly administered 0.25% methylcellulose without FF (control group). Rats in both groups received the treatment once daily by oral gavage for 4 weeks.

Tissue collection. On day 29, rats were anesthetized by ether and sacrificed by exsanguination. The skeletal muscle of the rats was dissected and snap frozen in liquid nitrogen. Skeletal muscle samples were stored at -70˚C until use.

RNA extraction and analysis. Total RNA was isolated from rat skeletal muscle according to a previously described method, with minor modifications (31). Briefly, dissected muscle tissue was frozen in liquid nitrogen, ground to powder, homogenized with a Polytron homogenizer and finally extracted with Trizol reagent (Invitrogen, MD, USA). After ethanol precipitation, total RNA was dissolved in DEPC-treated water for direct use or stored in 70% ethanol at -70˚C. Following isolation, RNA quantity was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA). To determine the purity and quality of the RNA, samples were electrophoresed on 1% agarose gel and visualized by UV transillumination after ethidium bromide staining.

Semiquantitative RT-PCR. SqRT-PCR was performed in order to roughly assess changes in the rSema6B mRNA level after FF treatment. Reverse transcription was performed using an oligo (dT)$_{12-18}$ first-strand primer. For PCR amplification, two pairs of specific primers (Table I) were used, one pair for rSema6B and another pair for β-actin as an internal control.

The primers were designed using Primer Premier 5.0 (Biosoft International). A Blast search was conducted to confirm that the primer pairs only matched the sequence of the target genes. RT-PCR amplification was performed using Ready-To-Go RT-PCR Beads (GE Healthcare, Amersham Biosciences, Germany) containing Moloney murine leukemia virus reverse transcriptase and Taq DNA polymerase. The reaction mixture contained 2 μg total RNA, oligo (dT) first-strand primer, one pair of specific primers and DEPC-treated water in a total volume of 33 μl. A two-step protocol for RT-PCR was performed using a thermocycler (GeneAmp® PCR System 9700, Applied BioSystems) as follows: first-strand synthesis at 37˚C for 60 min, initial denaturation at 95˚C for 3 min, denaturation for 35 (rSema6B) or 18 (β-actin) cycles at 95˚C for 30 sec, annealing at 60˚C for 3 sec, elongation at 72˚C for 30 sec, and a final extension at 72˚C for 10 min. Preliminary experiments confirmed that both RT-PCR reactions were stopped within the linear phase of the PCR amplification. Each sample was electrophoresed on 2% agarose gel and visualized by UV transillumination after ethidium bromide staining. Fluorescence intensity was quantified using a Gel Doc 2000 system (Bio-Rad).

Quantitative real-time PCR. Total RNA (2 μg from each sample) was reverse transcribed using the M-MLV RT First-Strand Synthesis System (Invitrogen) and oligo (dT)$_{12-18}$ primer according to the manufacturer's instructions. Real time PCR reactions for the monoplex amplification of rSema6B (target gene) or β-actin (internal reference gene) were conducted using 2 μl cDNA, 300 nM of each primer pair (Table I), 1X PCR buffer, 3 mM MgCl$_2$, SYBR Green I (1:7000; Invitrogen), 200 nM of each dNTP and 1 unit of GoTaq Hotstart Taq poly-merase (Promega), for a final volume of 25 μl. Each reaction was run in duplicate. Real time PCR analysis was performed using a Quantica instrument (Techne, England) with the following program: enzyme activation at 95˚C for 3 min, dena-turation 95˚C for 40 cycles of 15 sec, annealing at 56˚C for 20 sec, and elongation at 72˚C for 35 sec. Fluorescence acqui-sition was performed in the SYBR Green channel at the end of each elongation step. After PCR, melt curve analyses were performed to control specific amplification. Threshold cycles (Cts) were automatically determined by the software using the 'fit point' method (2 fit points set up), and the average Ct value of every duplicate reaction was calculated.

Changes in the rSema6B mRNA level induced by FF treatment were determined using the ΔΔCt method (32). First, ΔCt values (Ct$_{rSema6B}$ - Ct$_{β-actin}$) were determined for the treated and non-treated samples. Then, the ΔΔCt was calculated as the difference between these ΔCts as follows: ΔΔCt = (Ct$_{rSema6B}$ -Ct$_{β-actin}$) (treatment group) - (Ct$_{rSema6B}$ - Ct$_{β-actin}$) (control group). The relative fold change (R) in rSema6B mRNA quantity in the treated samples was calculated using the equation: R = $2^{-ΔΔCt}$.

Extraction of membrane proteins and Western blotting. muscle tissue (500 mg) from FF-treated and non-treated rats was pulverized in liquid N$_2$. Proteins were extracted and the membrane-enriched fractions were prepared as previously described (20). Protein content in the membrane fractions was determined according to a previously reported method (33). Protein samples were fractionated by 12% SDS-PAGE and

Table I. Sequences of primers used in sqRT-PCR, qPCR, and ChIP assays.

| Gene	Technique	Sequence (5'-3') sense	Sequence (3'-5') antisense	Tm (˚C)	Product size (bp)
rSema6B	SqPCR	GAGTGGGGAAG CCACGTCTAC	GGGTCTTTACAAAG TTGAGAATCTCGT	60	499
β-actin	SqPCR	GTGGGGCGCCC CAGGCACCA	CTCCTTAATGTCA CGCACGATTTC	54	540
rSema6B	qPCR	AAGGGCAAGCA AGAGGGTGAG	TTGGAGCCGCA CACGAAGAG	60	86
β-actin	qPCR	AGCGTGGCTAC AGCTTCACC	AAGTCTAGGGCA ACATAGCACAGC	60	85
rSema6B promoter region	ChIP	AGGGAGCCTTG CCTAGAATC	GACATGAGCCT ACTTCCTACGA	60	171
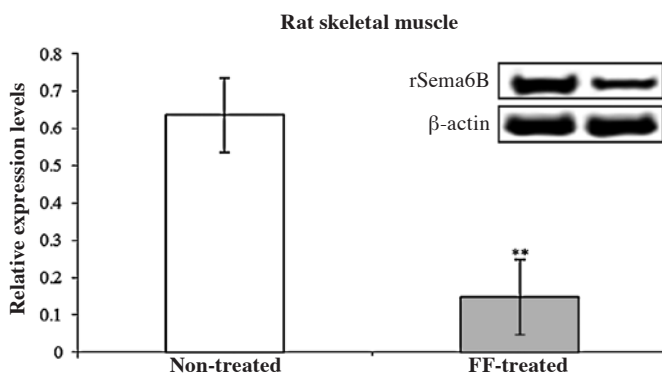
Figure 1. Decrease in the Sema6B mRNA level in rat skeletal muscle following FF treatment. Total RNA was extracted from skeletal muscle and subjected to semi-quantitative RT-PCR analysis. For rSema6B, a pair of primers amplified the expected 499-bp fragment. Panels were normalized against the 540-bp β-actin PCR fragment. Amplified DNA fragments were fractionated on a 2% agarose gel and stained by ethidium bromide. The ratio of the relative light units (RLUs) between rSema6B and β-actin signals was determined. The bar graph represents the mean ± SD of 3 independent experiments.

transferred onto paper membrane filters. Immunoreactivity was detected using an antibody raised against rSema6B (diluted 1:1000) (20). β-actin was used as an internal standard and detected by a specific antibody (diluted 1:5000). Visualization of specific protein bands was carried out using the BM chemiluminescence kit according to the manufacturer's protocol.

In silico analysis of rSema6B promoter. The genomic sequence of rSema6B (GenBank accession number AB000776) was downloaded from NCBI (ncbi.nlm.nih.gov), and a 2-kb fragment of the promoter region upstream of the transcription start site was scanned for putative PPREs using NUBIScan software (nuclear receptor binding site scanner) (34).

Chromatin immunoprecipitation (ChIP) assay. The binding of PPARα to the rSema6B promoter was performed according to the protocol described in the commercially available ChIP-IP™ Express kit (Active Motif, USA) as follows:

skeletal muscle from FF-treated rats was chopped into small pieces and treated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and gDNA. Tissues were then homogenized in 1% SDS lysis buffer, and 350 μl lysate of each group was sonicated in a sonicator (Sartorius, Goettingen, Germany). Immunoprecipitation was performed using anti-PPARα (H-98) antibody (Santa Cruz) or with IgG alone as a negative control. Immune complexes were recovered using magnetic agarose beads. Precipitated gDNAs were analyzed by monoplex PCR amplification for 32 cycles using primers flanking the putative PPRE in the rSema6B promoter or primers located about 6 kb upstream of the glyceraldehyde-3-phosphate dehydrogenase GAPDH promoter as a negative control. Aliquots of sheared DNA from tissue lysates that had not been immunoprecipitated were also included in the PCR reactions (input) to adjust for differences in the amount of gDNA in the different tissue samples. PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide.

Statistical analysis. Densitometric analyses of sqRT-PCR and Western blotting assays were carried out using a Gel Doc 2000 system. The data represent the mean ± SD of three independent experiments. The statistical significance of differences between data from the sqRT-PCR and Western blotting analyses were assessed using the Student's t-test. The acceptable level of significance was $P<0.05$.

Results

Effect of FF on rSema6B mRNA level. The effects of FF on the rSema6B mRNA level were determined in rat skeletal muscle by semi-quantitative RT-PCR. As shown in Fig. 1, FF treatment induced a significant decrease (of ~5-fold) in the Sema6B mRNA level.

Quantification of the change in rSema6B gene expression following FF treatment. Real-time quantitative PCR analysis was used to confirm and quantify the observed decrease in rSema6B gene expression in response to treatment with FF.
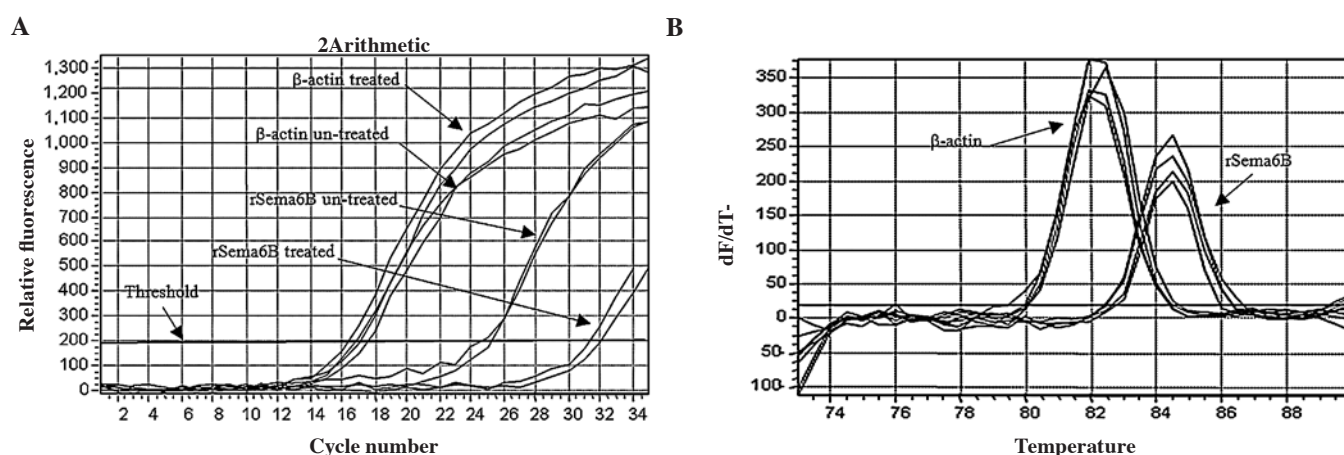
A

B

Figure 2. RT-PCR quantification of rSema6B gene expression after FF treatment. (A) Real-time PCR amplification curves and (B) subsequent melting analysis of rSema6B and β-actin genes. Total RNA was isolated from the skeletal muscle of FF-treated and non-treated rats. RNA was reverse transcribed and cDNA was amplified in duplicate PCR reactions (see Materials and methods). FF treatment increased rSema6B Ct values (threshold cycles) by about 6 cycles, whereas β-actin (reference gene) Ct values remain unchanged after FF treatment. Using the ΔΔCt method (32) to quantify rSema6B down-regulation, the 6 cycle delay observed in the rSema6B amplification curves corresponds to a 73.5-fold decrease in rSema6b mRNA.

Table II. ΔΔCt method comparing relative expression determined by real-time PCR between treatments.

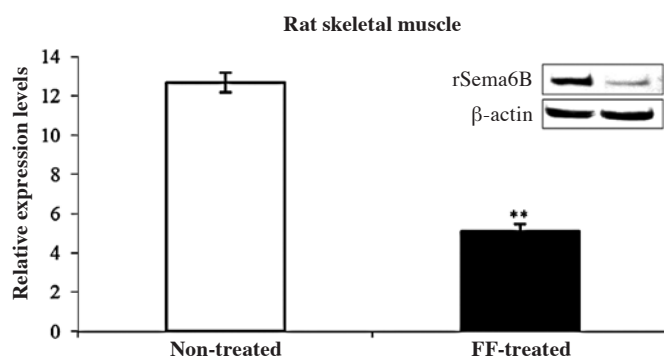|	Ct		Ct (mean)	ΔCt	ΔΔCt	R
Control				8.3		
β-actin	16	16.3	16.2			
rSema6B	24	25	24.5			
					+6.2	$2^{6.2}$	73.5-fold↓
Treated				14.5		
β-actin	16.5	17	16.7			
rSema6B	31	31.5	31.2			

Rat skeletal muscle

Figure 3. Effect of FF on rSema6B protein content in rat skeletal muscle. Rats were treated with FF for 4 weeks. Total protein was extracted from skeletal muscle and submitted to Western blotting (see Materials and methods). Blots were first probed with the polyclonal antibody raised against rSema6B, then with a polyclonal anti-β-actin antibody. The intensities of the bands were quantified and the ratios between the intensities of the rSema6B and β-actin signals were determined. FF treatment decreased rSema6B protein content in rat skeletal muscle by ~2.5-fold. The bar graph represents the mean ± SD of 3 independent experiments. *P<0.05 vs. the control.

Total RNA was isolated from the skeletal muscle of FF-treated and non-treated rats. As shown in Fig. 2, the rSema6B amplification curve of treated rats crossed the threshold line about 6 cycles after the rSema6B amplification curve of non-treated rats. As shown in Table II, the expression of the rSema6B gene was down-regulated by 73.5-fold after FF treatment.

Effect of FF on rSema6B protein content. Western blotting analysis revealed that FF treatment resulted in a marked reduction (of ~2.5-fold) in Sema6B protein content in rat skeletal muscle (Fig. 3).

In silico-analysis of rSema6B for the existence of putative PPREs. In order to define the PPRE of the rSema6B promoter potentially responsible for the observed PPARα-induced down-regulation in Sema6B protein content, a 2-kb fragment upstream of the rSema6B start codon was analyzed using NUBIScan software. Using a cutoff value of P≤0.015, this program revealed only one single putative PPRE situated -1157 bp (upstream of the ATG) on the sense strand. This putative PPRE (AGAGCAgAGGTCA) shows high similarity to the consensus PPRE (AGGTCAaAGGTCA), termed DR-1 (direct repeat 1), which is known the literature (35).

ChIP assays. To confirm that PPARα binds to the identified potential PPRE in the rSema6B promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed using an anti-PPARα antibody and anti-Rabbit IgG as a negative control. As shown in Fig. 4, a primer pair flanking the putative
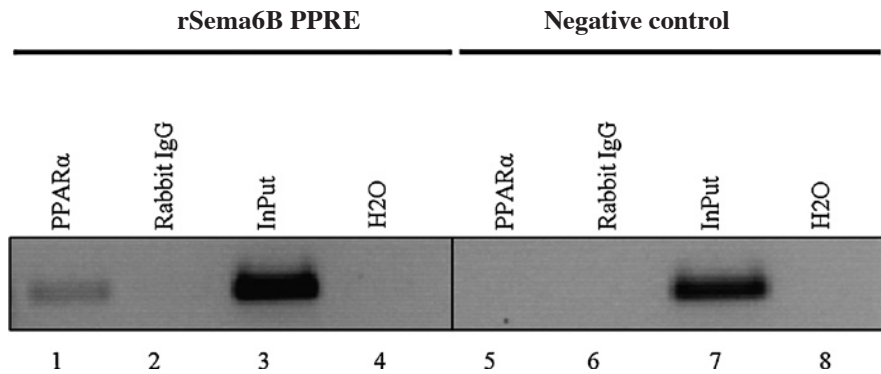
Figure 4. ChIP assay of the rSema6B promoter. Rats were treated with FF and skeletal muscle were processed for the ChIP assay. Soluble chromatin was immunoprecipitated with preimmune rabbit IgG (lanes 2 and 6) or anti-PPARα antibody (lanes 1 and 5). Immunoprecipitates were subjected to PCR using a primer pair flanking the putative PPRE in the rSema6B promoter. As a negative control, a second pair of primers were used to amplify another genomic region that was not expected to interact with PPARα. PCR was also performed with total chromatin input (lanes 3 and 7).

PPRE amplifies a specific PCR fragment from anti-PPARα immunoprecipitated chromatin, but fails to amplify the same amplicon from anti-Rabbit IgG immunoprecipitated chromatin. Furthermore, a primer pair situated 6 kb upstream of the putative PPRE fails to amplify any fragment, regardless of the antibody used. These results suggest that the identified PPRE binds PPARα *in vivo*.

Discussion

Beside their established role as guidance molecules for neurons and migrating cells (12), a role of semaphorins in skeletal muscle has been suggested by several reports. For example, expression of the axon repellent hSema3A is markedly up-regulated in satellite cells of resident myogenic stem cells when treated with hepatocyte growth factor (HGF), established as an essential cue in muscle fiber growth and regeneration (36). In addition, *in vivo* hSema3A was shown to be up-regulated in the differentiation phase of satellite cells isolated from regenerating muscles after crush injury (36,37). hSema3A was also shown to be differentially expressed in terminal Schwann cells (TSCs) in various populations of muscle fibers. This expression may suppress nerve terminal plasticity at specific neuromuscular synapses (38). In addition, it was shown that Sema4C is dramatically induced not only during the differentiation of C2C12 mouse myoblasts, but also during injury-induced skeletal muscle regeneration, suggesting novel functional potentialities for mSema4C in promoting terminal myogenic differentiation (39).

On the other hand, it has been shown that both human Sema6C and Sema6D are expressed in the skeletal muscle, and that the expression of both semaphorin genes is down-regulated following denervation (40). Furthermore, it was found that hSema6C is expressed in extrasynaptic as well as in perisynaptic regions of muscles, and that the protein is concentrated at neuromuscular junctions, suggesting a role of hSema6C in neuromuscular communication (41).

Human Sema6B is highly expressed in the brain and skeletal muscle and expressed at lower levels in a variety of other tissues (18). Like Sema6A, Sema6B was recently shown to inhibit the growth of sympathetic ganglion axons (42). In addition, hSema6B transcript was shown to be down-regulated in two different human glioblastoma cell lines (T98G and A172) upon prolonged treatment with all-trans-retinoic acid or PPAR ligands (18-20).

In previous studies, we demonstrated that the PPARα agonist FF induces a significant decrease in the mRNA level and protein content of Sema6B in glioblastoma (T98G) and breast cancer (MCF7) cell lines (19,20). In this study, we investigated the effect of FF on Sema6B expression in rat skeletal muscle. Our qRT-PCR and Western blotting experiments clearly revealed that the expression of the rSema6B gene is down-regulated by FF treatment. Furthermore, our ChIP results demonstrated that the PPRE predicted by the NUBIScan software in the rSema6B promoter region (-1157 upstream of the start codon) binds to PPARα *in vivo*. These results are in full agreement with the data from the above mentioned studies (19,20).

In conclusion, our data suggest that the expression of Sema6B in rat skeletal muscle is regulated by PPARα. The biological significance of the control exerted by PPARα in the regulation of rSema6B expression warrants further investigation. Additional studies with other semaphorin genes will validate and expand our results. In view of the implication of several members of the semaphorin class 6 in the regulation of skeletal muscle regeneration, we conclude that rSema6B may also play a role in this process.

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