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Lithocholic acid promotes skeletal muscle regeneration through the TGR5 receptor

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Abstract
Lithocholic acid (LCA) is a classical secondary bile acid formed by the metabolism of gut microbiota. The TGR5 receptor (also known as G protein-coupled receptor 1, GPBAR1) is an important bile acid membrane receptor that mediates a variety of metabolic processes in vivo. In recent years, most studies have focused on the role of bile acid receptors in the intestine and liver. However, there are few reports on its effect on skeletal muscle development, and the specific mechanism is not clear.
Therefore, it is necessary to investigate the mechanism of the TGR5 receptor in the regulation of skeletal muscle development. The results demonstrate that muscle injection with LCA significantly reduces the necrosis rate of injured muscle and improves muscle injury. Moreover, treatment of C2C12 cells with LCA significantly increases AKT/mTOR/FoxO3 phosphorylation through the TGR5 receptor, enhances MyoG transcription and reduces FBXO32 transcription. These findings indicate that LCA can activate the TGR5/AKT signaling pathway, inhibit protein degradation and promote protein synthesis to enhance the myogenic process and promote skeletal muscle regeneration.

**Keywords:** muscle injury, TGR5 receptor, lithocholic acid, proliferation, differentiation

**Introduction**

Bile acids (BAs) are metabolites of cholesterol in the liver; according to their structure, bile acids are divided into free bile acids and bound bile acids. Moreover, bile acids are also classified by source, including primary and secondary bile acids. Among them, the secondary bile acids are mainly deoxycholic acid (DCA) and LCA. Bile acid is an emulsifier that can emulsify lipids in the digestive tract into lipid drops or chylomicrons (CMs). Finally, it can promote the digestion and absorption of lipids and fat-soluble vitamins in the small intestine [1,2]. In addition, as important signal regulatory molecules, bile acids can activate bile acid receptors and participate in bile acid metabolism, glucose and lipid metabolism, energy metabolism and immune regulation [3,4]. In addition, bile acids interact with intestinal microorganisms to regulate energy metabolism, lipid metabolism, cholesterol and bile acid homeostasis [5–7].

Peripheral tissues mainly sense bile acid through the farnesoid X receptor (FXR) or Takeda G protein-coupled receptor 5 (TGR5), thus participating in a series of metabolic processes in the body. Two receptors that can be activated by bile acid are the nuclear receptor FXR and the membrane receptor TGR5 [8]. Both of them are expressed in a variety of tissues, such as the liver and small intestine. It has been reported that the FXR agonist fexaramine can significantly improve the blood glucose level in vivo and reduce the weight gain induced by diet [7]. Meanwhile, systemic FXR-knockout mice show significant changes in glucose tolerance and body weight.
FXR can mediate chenodeoxycholic acid (CDCA) to promote the proliferation and differentiation of mouse embryonic stem cells [11]. Additionally, it has been confirmed that the level of bile acid in mice increases and causes liver damage, leading to liver steatosis, inflammation and fibrosis after knocking out the FXR receptor in mouse livers [12].

Relevant studies have shown that TGR5 agonists ameliorate diabetes-induced retinal microvascular dysfunction in vivo and inhibit the effect of TNF-α on endothelial cell proliferation, migration and permeability in vitro by regulating the ROCK signaling pathway [13]. Ursodeoxycholic acid (UDCA) suppresses the malignant progression of colorectal cancer by regulating TGR5-YAP signaling from primary bile acids and partial secondary bile acids. Therefore, it is important to maintain normal intestinal bile acid metabolism in cancer patients [14]. TGR5, the bile acid G-protein coupled receptor highly expressed in biliary epithelial cells, protects the liver against BA overload through the regulation of biliary epithelium permeability [15]. To date, LCA and taurolithocholic acid (TLCA) have been described as the most potent endogenous TGR5 ligands [16,17]. It has been reported that activation of TGR5 by lithocholic acid induces AQP2 expression and intracellular trafficking in rat IMCD cells via a cAMP-protein kinase A signaling pathway. In mice with nephrogenic diabetes insipidus (NDI), dietary supplementation with LCA markedly decreased urine output and increased urine osmolality while significantly upregulating aquaporin 2 (AQP2) expression in the kidney inner medulla. However, supplementation with endogenous FXR agonists had no effect [18].

The TGR5 receptor, as the most intensively studied membrane-bound G protein-coupled receptor, is expressed in many tissues in vivo, and a small amount of the TGR5 receptor is found in muscle [16]. Muscle fibers isolated from the muscle of TGR5−/− mice show atrophy, and cholic acid (CA) and DCA treatment induce an increase in ROS production and 4-HNE modification of proteins, causing an increase in ubiquitin-proteasome system (UPS) and autophagy components associated with muscle atrophy. Therefore, the muscle fiber diameter and myosin heavy chain (MyHC) protein expression level increased significantly, while Atrogin-1 and MuRF-1 protein expression levels decreased significantly [19]. Previous reports have shown that knockout of the TGR5 receptor in mouse muscle results in a decrease in muscle mass and muscle strength, while the expression levels of hypertrophy-related genes and differentiation-related genes are also downregulated. However, activation of the
TGR5 receptor enhances the differentiation ability of muscle cells and promotes muscle hypertrophy [20]. Previous studies have indicated that increased circulating total bile acid level promote skeletal muscle hypertrophy and enhance skeletal muscle volume [21]. Clinical studies have revealed that tauroursodeoxycholic acid (TUDCA) improves insulin signal transduction in the liver and muscle of obese patients [22]. Dietary supplementation with 60 mg/kg and 80 mg/kg bile acids significantly increases the leg muscle weight of broilers [23]. This result suggested that bile acid receptors play an important role in the regulation of skeletal muscle development.

We previously found that in addition to the significant difference in the expression levels of myogenic factors between two breeds of female weaned piglets (Duroc and Laiwu), there were significant differences in the level of serum LCA and the expression level of bile acid receptors in skeletal muscles. Correlation analysis showed that the expression levels of myogenic factors were markedly correlated with that of TGR5. Previous studies have shown that total bile acids are associated with skeletal muscle volume, and 12 hydroxysterol bile acids (including deoxycholic acid) are associated with skeletal muscle volume reduction [21]. Dietary supplementation with phytosterol ester (PE) can affect bile acid transport and lipid metabolism between the mother and fetus and may promote muscle development in the offspring through the action of bile acid receptors [24,25].

Lithocholic acid (LCA) is a classical secondary bile acid formed by the metabolism of gut microbiota. According to relevant research reports, LCA impaired gut barrier function by enhancing the EGFR-Src pathway and then affected gut health [26]. It has also been found that LCA induces the stromal to epithelial transformation of breast cancer cells through the activation of TGR5, reduces cancer cell proliferation and VEGF production, reduces the invasive and metastatic potential of primary tumors, and increases the antitumour immune response [27]. Currently, it has been reported that treatment with live P. distasonis (LPD) dramatically alters the bile acid profile with elevated LCA to decrease weight gain and hyperglycaemia [28]. Interestingly, intramuscular injection of taurocholic acid (TLCA) can activate the TGR5 receptor in mature muscle tissue, resulting in muscle hypertrophy and muscle strengthening [20]. Maruyama et al. [29] also found that female TGR5−/− mice showed faster weight gain than wild-type mice under high-fat diet conditions. Body composition analysis revealed that female TGR5−/− mice had a higher fat content.
In the present study, we explored the effect of LCA on skeletal muscle regeneration and its mechanism through relevant experiments. We hope that the results of this study can provide an experimental basis for studying the role of bile acids in skeletal muscle.

**Materials and Methods**

**Animals and experimental design**

All experiments were performed following the Guidelines for Animal Care and Handling approved by China Agricultural University (Beijing, China). Male C57BL/6J mice (3 weeks old) were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). After being fed with a normal diet for 4 weeks (7 weeks old), 15 mice were divided into three groups according to the three time points of the experimental treatment (3 d, 5 d, and 7 d), with 5 mice in each group. Then, 100 μL of cardiotoxin (CTX, 10 μM in PBS; Sigma, St Louis, USA) was injected into the gastrocnemius muscle of each leg. Gastrocnemius muscle was harvested at three time points.

After being fed with a normal diet for 4 weeks (7 weeks old), 20 mice were randomly divided into two groups, with 10 mice in each group. Then, their gastrocnemius muscle was injected with CTX (100 μL per leg). On the third day after CTX injection, the control group mice were injected with normal saline (100 μL per leg, 10% BSA). The experimental group mice were injected with LCA (100 μL per leg, 0.4 mg/100 μL in saline with 10% BSA; MCE, Shanghai, China). After four days, the gastrocnemius muscle was harvested.

**Frozen section preparation**

GAS muscle was harvested, equilibrated in a cryosectioning machine for 30 min, trimmed and embedded for longitudinal cutting. Finally, frozen sections were obtained at a thickness of 10 μm.

**Treadmill exercise**

Seven-week-old LCA-injected mice and saline-injected mice were acclimated to running on a treadmill (Taimeng Software Co., Ltd, Chengdu, China) at a 10% incline for 60 min. Running speed was set at 8 m/min for the first 5 min and increased to 12 m/min for the next 5 min. Subsequently, the speed was increased to 16 m/min for 20
min, and finally, 30 min was set to 20 m/min. Treadmill running was continued until mice were exhausted (they remained on an electric stimulus grid for 6 s) or completed a 60-min running program.

**Cell culture and treatment**

C2C12 cells (ATCC, Manassas, USA) were cultured in growth medium (DMEM; Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 IU/mL ampicillin-streptomycin (Gibco) in a constant-humidity atmosphere (5% CO₂ and 37°C). For differentiation into myotubes, C2C12 cells were plated in 12-well plates at a density of 5×10⁴ cells/well. When C2C12 cells reached 80% confluence, the medium was changed to differentiation medium supplemented with 2% horse serum (HyClone, Logan, USA) and cultured for another 3 days. Then, C2C12 cells were treated with 50 μM LCA (MCE) for 3 days. After treatment, C2C12 cells were harvested for western blot analysis and qRT-PCR.

**CCK8 assay**

C2C12 cells were plated in 96-well plates at a density of 2×10³ cells/well and cultured in growth medium for 24 h. Then, C2C12 cells were treated with different concentrations of LCA for different time intervals. After treatment, the medium was changed to growth medium without LCA, and then a 10 μL CCK8 reagent (Absin, Shanghai, China) was added to each well and incubated for 1.5 h at 37 °C. Finally, the absorbance was measured at 450 nm using a multifunctional microplate reader.

**EdU incorporation assay**

After addition of 100 μL EdU medium (50 μM) to each well in the 12-well plate and incubation for 2 h, the medium was discarded, and the cells were washed twice with PBS. Cells in each well were fixed with 50 μL of cell fixative (PBS with 4% paraformaldehyde) for 30 min at room temperature, and then 50 μL of glycine (2 mg/mL) was added to each well and incubated for 5 min. Cells in each well were washed with 100 μL PBS, penetration with 100 μL of penetrant (PBS with 0.5% TritonX-100) for 10 min. After wash with PBS, 100 μL of 1×Apollo reaction solution was added to each well and incubated for 30 min, followed by 2–3 times wash with 100 μL PBS and 1–2 times wash with 100 μL of methanol. Finally, 100 μL of 1× Hoechst solution was added to each well and stained for 30 min, and fluorescence images were captured.
under a fluorescence microscope after 1–3 times wash with PBS.

**LDH cytotoxicity assay**

C2C12 cells were plated in 96-well plates at a density of $2 \times 10^3$ cells/well. Then, C2C12 cells were treated with different concentrations of LCA for 24 h. After 1 h of incubation with PBS, C2C12 cells were collected and tested using the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

**siRNA experiments**

C2C12 cells were transfected with TGR5 siRNA (GenePharma, Shanghai, China) according to the manufacturer’s instructions. The siRNA sequences are shown as follows: siTGR5: 5′-CCUCUACCUGGAAGUUAUTT-3′ (sense); 5′-AUAAACCUCCAGGUAGAGGTT-3′ (antisense) and siCtrl: 5′-UUUCUGAAGCUGAGCAGUTT-3′ (sense); 5′-ACGUGACACGUUCGAGAATT-3′ (antisense). Twenty-four hours after transfection, cells were treated with LCA treatment. After treatment, the medium was collected for protein extraction according to the manufacturer’s protocols (Thermo Fisher Scientific, Rockford, USA).

**Quantitative RT-PCR**

Total RNA was extracted using a general RNA extraction kit (Magen, Guangzhou, China) according to the manufacturer’s instructions. Total RNA was reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA) and subjected to quantitative RT-PCR analysis using SYBR Green (EZBioscience, Roseville, USA) on a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The primers used for the PCR analysis were synthesized by Sangon (Shanghai, China) and the sequences are shown in Table 1.

**Western blot analysis**

Gastrocnemius muscle was homogenized in radioimmunoprecipitation assay (RIPA) buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then centrifuged to collect the supernatant. C2C12 cells were washed with PBS and lysed in RIPA lysis buffer containing 1 mM PMSF, followed by centrifugation to collect the
supernatant. The protein concentrations in the supernatant were determined using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific). Then, 20 μg of protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked in 6% skim milk powder dissolved in TBST for 2 h at room temperature, followed by incubation with primary antibodies overnight at 4°C. Subsequently, PVDF membranes were washed with TBST and incubated with appropriate HRP-conjugated secondary antibodies (goat anti-rabbit or mouse; Jackson, West Grove, USA) for 1 h at room temperature. After wash with TBST, ECL reagent (Millipore, Billerica, USA) was used to visualize protein bands. The following primary antibodies were used: mouse anti-FXR, myogenin and MyoD (Abcam, Cambridge, UK), rabbit anti-FBXO32 and anti-MuRF1 (Abclonal, Shanghai, China), rabbit anti-PCNA and cyclin D1 (Zhengneng Biotechnology, Chengdu, China), rabbit anti-TGR5 and eMyHC (Novus, Littleton, USA), rabbit anti-p-AKT, p-mTOR, p-S6, p-FoxO3 and AKT, mTOR, S6, FoxO3 (CST, Beverly, USA), and rabbit anti-β-tubulin (Abcam).

**Immunofluorescence staining**

Muscle sections were fixed in 4% paraformaldehyde and washed with PBS. Samples were blocked in blocking buffer (5% goat serum) for 2 h at room temperature, followed by incubation with primary antibodies in blocking buffer at 4°C overnight. Subsequently, the samples were washed with PBS and stained with the appropriate fluorescently-labelled secondary antibodies (FITC or Alexa Fluor 555; Jackson) for 1.5 h at room temperature. After extensive wash with PBS, the samples were mounted with anti-fluorescence quenching sealing agent and examined under a fluorescence microscope. The following primary antibodies were used: mouse anti-eMyHC (Novus), rabbit anti-laminin (Sigma).

Cultured cells were fixed in 4% paraformaldehyde and permeabilized for 10 min with 0.4% Triton X-100 in PBS. Samples were blocked in blocking buffer (5% goat serum) for 30 min at room temperature, followed by incubation with primary antibodies in blocking buffer at 4°C overnight. Subsequently, the samples were washed with PBS and stained with the appropriate fluorescently-labelled secondary antibodies (FITC or Alexa Fluor 555) for 1.5 h at room temperature. After wash with
PBS, DAPI was used to stain the nuclei for 2 min. Finally, cells were examined under a fluorescence microscope. The following primary antibodies were used: mouse anti-eMyHC (Novus).

**Necrotic fiber analysis**

Necrotic fibers refer to broken break links between muscle fibers and no complete cross section. Images of the necrotic fibers were acquired by tissue immunofluorescence staining and immunofluorescence microscopy. The numbers of muscle fibers as well as the number of normal fibers were calculated by ImageJ, and finally, the percentage of necrotic fibers was calculated using the following formula:

\[
\text{Necrotic fibers (\%)} = \frac{(A - B)}{A} \times 100\%
\]

where A is total muscle fiber, and B is normal muscle fiber.

**Statistical analysis**

The results were analyzed and figures were drawn using GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, USA). Data are presented as the means ± standard errors of the means (SEM). Student’s t test was used to compare the differences between groups. One-way ANOVA was used to compare the differences among multiple groups. Statistical significance was defined as \( P<0.05 \).

**Results**

**LCA promotes the healing process of CTX-induced skeletal muscle injury**

To address this concern, we injected cardiotoxin (CTX) into Gastrocnemius muscle, resulting in Gastrocnemius muscle injury, and found that Gastrocnemius muscle injury was the most serious at 3 d postinjury (Figure 1A,B). Therefore, we injected LCA into Gastrocnemius muscle at 3 d postinjury (Figure 1C). Although LCA did not affect the exercise ability or muscle quality of mice (Figure 1D–F), we found that LCA significantly reduced the necrosis rate of Gastrocnemius muscle (Figure 1G). It seems that LCA has a potential effect on skeletal muscle.

**LCA promotes the proliferation process of injured skeletal muscle and C2C12 cells**

When the injured skeletal muscle is being repaired, it is mainly the continuous proliferation and differentiation of muscle cells that promote muscle regeneration and
hypertrophy. First, we investigated whether LCA could affect muscle cell proliferation during the healing process of injured skeletal muscle. Statistical analysis showed that the protein expression levels of PCNA and cyclin D1 were upregulated significantly (Figure 2A,B). Moreover, CCK-8 assay indicated that cell viability was increased significantly in cells treated with 8 μM and 10 μM LCA. Treatment with greater than 10 μM LCA reduced cell viability (Figure 2C). The same concentration of LCA was used to treat C2C12 cells. LDH assay showed that 40-100 μM LCA was toxic to cells, while 2–10 μM LCA was nontoxic to cells (Figure 2D). Western blot analysis showed that the protein level of PCNA was upregulated significantly after treatment with 10 μM LCA (Figure 2E,F). The EdU incorporation assay showed that 10 μM LCA significantly increased the percentage of EdU-positive C2C12 cells, indicating that 10 μM LCA can increase the percentage of C2C12 cells in the S phase of the division phase (Figure 2G). These results suggest that LCA promotes the proliferation of injured muscle cells and C2C12 cells, further improving skeletal muscle injury caused by CTX injection.

LCA promotes the differentiation process of injured skeletal muscle and C2C12 cells

In injured muscle, statistical analysis showed that the number of eMyHC+ fibers was increased significantly in mice injected with LCA (Figure 3A). Western blot analysis and qRT-PCR results showed that the expression levels of MyoG and MyoD were markedly increased (Figure 3B–D). Meanwhile, the results in C2C12 cells showed that 100 and 200 μM LCA were toxic to C2C12 cells (Figure 3E), and the differentiation index and the number of MyoG+ cells were significantly increased in C2C12 cells treated with 50 μM LCA (Figure 3F,G). At the same time, the protein level of MyoG was significantly increased (Figure 3H,I). These results indicate that LCA can promote cell differentiation.

LCA affects protein metabolism of injured skeletal muscle and C2C12 cells

More importantly, our results showed that S6/FoxO3 phosphorylation levels were increased significantly in mice injected with LCA (Figure 4A,B). However, MuRF1 protein and mRNA expression levels were significantly reduced (Figure 4C–E). Moreover, AKT/mTOR/FoxO3 phosphorylation levels were significantly upregulated in C2C12 cells, while degradation gene expressions associated with muscle necrosis
FBXO32 protein levels were significantly reduced (Figure 4F–I). These results indicate that LCA promotes protein synthesis, inhibits protein degradation, and finally affects muscle cell protein metabolism.

**LCA promoted cell differentiation through the TGR5 receptor**

In this study, we found that TGR5 receptor protein levels were markedly increased in skeletal muscle injected with LCA and in C2C12 cells treated with 50 μM LCA (Figure 5A–D). In C2C12 cells stably expressing TGR5 siRNA (siTGR5), the TGR5 receptor protein level was effectively downregulated in siTGR5 C2C12 cells (Figure 5E,F). After silencing of the TGR5 receptor, the effect of increased MyoG and MyoD expression levels was reversed (Figure 5G,H). At the same time, the effect of reduced FBXO32 protein expression levels and increased phosphorylation levels of FoxO3 were reversed (Figure 5I,J). The effect of increased AKT and mTOR phosphorylation levels was also reversed (Figure 5K,L). Taken together, these results indicate that LCA promotes cell differentiation through the TGR5/AKT signaling pathway.

**Discussion**

In a preliminary study, we found that the repair process of CTX-treated skeletal muscle and cell proliferation were promoted after treatment with a certain dose of LCA. Skeletal muscle atrophy is characterized by decreased myofiber protein expression, increased muscle-specific E3-ubiquitin ligase expression, and loss of muscle weight and function [30,31]. Abrigo et al. [19] used a culture of muscle fibers isolated from WT and TGR5−/− mice to evaluate the participation of TGR5 in muscle atrophy induced by DCA and CA. The results showed that DCA and CA decreased the fiber diameter and MHC protein levels, and there was an increase in atrogin-1, MuRF-1, and oxidative stress in WT fibers. However, the absence of TGR5 in fibers abolished all these effects induced by DCA and CA. Ischemia/reperfusion (I/R) injury reduced cell proliferation, triggered inflammation, and promoted cell apoptosis and necrosis. Acetoacetate (AA) is an important ketone body used as an oxidative fuel to supply energy for the cellular activities of various tissues, including the brain and skeletal muscle, promotes muscle cell proliferation *in vitro* and enhances muscle regeneration *in vivo*, ameliorates the dystrophic muscle phenotype of Mdx mice, and promotes C2C12 cell proliferation [32]. Li et al. [33] found that overexpression of TGR5 significantly improved cell proliferation and alleviated apoptosis and necrosis.
rates and the activities of caspase-3 and Bax protein expression levels by exploring the underlying molecular mechanism of TGR5 in hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury in vitro. Interestingly, we found that the expression levels of MuRF1 and FBXO32 were decreased and the number of eMyHC+ fibers was increased after the injection of LCA into atrophic muscle. Meanwhile, the necrosis rate of Gastrocnemius muscle is also reduced. In addition, activation of the TGR5 receptor by obacuone and ursolic acid was found to increase muscle mass and promote muscle hypertrophy in mice [34]. It was also found that many regulatory pathways are involved in liver regeneration after partial hepatectomy (PH) to initiate growth, protect liver cells, and sustain the functions of the remnant liver. Péan et al. [35] confirmed that after PH, TGR5-knockout mice exhibited slower liver mass restoration and severe hepatocyte necrosis, prolonged cholestasis, exacerbated inflammatory response, and delayed regeneration. Furthermore, we also found that the expression level of proliferative proteins was increased significantly in skeletal muscle cells and C2C12 cells. It has been reported that taurodeoxycholic acid (TDCA) could promote cell proliferation in the esophageal adenocarcinoma cell line FLO and human Baret’s cell line BAR-T [36]. Cassaburi et al. [37] found that low concentrations of CDCA, through TGR5-dependent CREB signaling activation, induced a significant increase in Cyclin D1 protein level and mRNA expression [37]. There are also some data showing that the TGR5-cAMP-PKA-PI3K-mediated pathway may play an important protective role in bile acid-induced modulation of choanocyte damage and cell proliferation [38]. Furthermore, it has been reported that bile acids are involved in the process of cell proliferation and programmed cell death (apoptosis) [39]. Based on our data, we suggest that LCA plays a potential role in promoting skeletal muscle injury repair and cell proliferation.

It has been reported that the TGR5 receptor may be involved in regulating the differentiation and metabolism of muscle cells [19]. Several previous reports have also confirmed that TGR5 receptor overexpression could enhance muscle cell differentiation [20]. Sasaki et al. [20] confirmed that activation of the TGR5 receptor could promote myocyte differentiation. As an important intracellular signaling molecule, AKT plays an important role in the process of cell proliferation and growth, leading to apoptosis. The results of a previous study showed that overexpression of TGR5 induced an increase in the level of p-AKT [33]. Zhu et al. [40] demonstrated that HDCA prevents LPS-induced microglial inflammation in vitro and in vivo, the
action of which was via regulating the TGR5/AKT/NF-kB signaling pathway. A previous study showed that administration of CA- and INT-777-supplemented diets upregulated TGR5 expression and activated AKT in the heart of mice [41]. It has been reported that treatment of mouse hypothalamus GT1-7 cells with TLCA or CDCA could activate TGR5 and significantly increase the expressions of intracellular signaling proteins, including p-AKT, p-STAT3 and SOCS3 [42]. Desai et al. [43] found that treatment of mouse cardiomyocytes (which express the membrane bile acid receptor TGR5) with potent natural TGR5 agonists and lithocholic acid activated AKT and inhibited GSK3β. Interestingly, Abrigo et al. [44] found that the decline in muscle function and contractibility induced by the DDC diet was dependent on TGR5 expression. Meanwhile, TGR5 dependence was also observed for the decrease in fiber diameter and sarcomeric proteins, as well as for the fast-to-slow shift in muscle fiber type. UPS (ubiquitin-proteasome system) overactivation, indicated by increased FBXO32 and muscle RING-finger protein-1 (MuRF-1) protein levels, was abolished in tibialis anterior muscles from TGR5‒/‒ mice. It has been reported that muscle fibers were isolated from wild-type (WT) and TGR5-deficient (TGR5−/−) male mice, and then treated with DCA and CA to isolate muscle fibers, the result showed that there was an increase in atrogin-1 and MuRF-1 in WT fibers. The absence of TGR5 in fibers abolished all these effects induced by DCA and CA [19]. The FoxO gene family (FoxO1, 3 and 4) is a downstream target gene of AKT, which mainly acts as a transcription factor to regulate the expressions of MuRF1 and FBXO32 [45]. MHC and troponin are targets of MuRF1 and FBXO32 and can be further degraded by the proteasome [46]. Muscle-specific loss of FoxO prevents protein degradation and autophagy [47]. Interestingly, we found that the expression levels of related differentiation genes were significantly increased in skeletal muscle cells and C2C12 cells. The phosphorylation levels of AKT and mTOR are significantly increased in C2C12 cells. Although the phosphorylation level of FoxO3 is markedly increased in skeletal muscle and C2C12 cells, the protein levels of degradation genes are significantly reduced. This result indicated that LCA could promote cell differentiation and affect the protein metabolism of muscle cells.

Sasaki et al. [20] showed that ATF6α activated by exercise regulates TGR5 transcription in skeletal muscle. It has been reported that the circulating level of GLP-2 is elevated, which can promote TGR5 activation [14]. Moreover, the
cotreatment results of LCA and siTGR5 showed that the effects of LCA on myogenic factors and degradation- and synthesis-related proteins all disappeared. Studies showed that TGR5-mediated cAMP production by OA was lost in siTGR5-transfected cells and in TGR5−/− mice [48]. Yasuda et al. [49] demonstrated that TGR5-specific siRNA suppresses the DCA-induced activation of EGFR and ERK1/2 and potentiates apoptosis in cancer cells. It has been shown that LCA more selectively activates the TGR5 receptor than other bile acids [50]. However, the molecular basis for its effects remains obscure. According to our research data, we surprisingly found that LCA may enhance the phosphorylation levels of AKT/mTOR/FoxO3, decrease FBXO32 expression levels, and upregulate MyoG expression levels by activating the TGR5 receptor.

In conclusion, LCA, as a classical secondary bile acid, can inhibit protein degradation, accelerate protein synthesis and cell differentiation by activating the TGR5/AKT signaling pathway, and ultimately promote injured skeletal muscle regeneration.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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**Figure legends**

**Figure 1. LCA promotes the healing process of injured skeletal muscles** (A) Schematic outline of CTX injection at the age of 7 weeks. (B) Immunofluorescence staining of injured mice at 3 d, 5 d and 7 d postinjury. n=5, scale bar=100 μm. (C) Schematic outline of LCA injection at 3 d postinjury. (D) Mice running training time. (E,F) Muscle weight. (G) Immunofluorescence staining of LCA injection at 3 d postinjury and the ratio of necrotic muscle fibers are shown on the right. n=6, scale bar=100 μm. Data are shown as the mean ± SEM. *P< 0.05; Student's t test.

**Figure 2. LCA promotes the proliferation of injured muscle cells and C2C12 cells** (A,B) Western blot analysis of Cyclin D1 and PCNA protein levels in injured muscle of control and LCA-injected mice at 5 d. n=8. (C) CCK-8 assay of different concentrations of LCA treatment for 24 h in proliferation medium. n=10. (D) LDH analysis of different concentrations of LCA treatment for 24 h proliferation medium. n=10. (E,F) Western blot analysis of Cyclin D1 and PCNA protein levels in C2C12 cells of the control and treatment with 10 μM LCA for 2 d in proliferation medium.
n=6. (G) Immunofluorescence images of EdU infiltration of C2C12 myoblasts measured by 10 μM LCA treatment. Analysis statistics of the percentage of value-added EdU positive cells in C2C12 cell treated with 10 μM LCA. n=6, scale bar=50 μm. Data are shown as the mean ± SEM. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Student’s t test, One Way ANOVA.

Figure 3. LCA promotes the differentiation of injured muscle cells and C2C12 cells
(A) Immunofluorescence analysis of eMyHC+ fibers in the gastrocnemius muscle of control and LCA-injected mice at 5 d. The percentage of area occupied by eMyHC+ fibers per field is shown on the right. n=6, scale bar=100 μm. (B,C) Western blot analysis of MyoD and MyoG protein levels in injured muscle of control and LCA-injected mice at 5 d. n=8. (D) MyoD and MyoG mRNA levels were measured in injured muscle of control and LCA-injected mice at 5 d. n=8. (E) LDH analysis of different concentrations of LCA treatment for 24 h in differentiation medium. n=6. (F) Immunofluorescence staining for eMyHC in C2C12 cells of control and 50 μM LCA treatment for 3 d in differentiation medium and then quantified for the differentiation index as percentage of nuclei in myosin heavy chain-positive cells is shown on the right. n=6, scale bar=100 μm. (G) Immunofluorescence staining for MyoG in C2C12 cells of control and 50 μM LCA treatment for 3 d in differentiation medium and the percentage of MyoG+ cells is shown on the right. n=6, scale bar=100 μm. (H,I) Western blot analysis of MyoD and MyoG protein levels in C2C12 cells of control and 50 μM LCA treatment for 3 d in differentiation medium. n=6. Data are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; Student’s t test, One Way ANOVA.

Figure 4. LCA affects protein metabolism in injured muscle cells and C2C12 cells
(A–D) Western blot analysis of protein turnover signaling pathway-associated protein levels in injured muscle of control and LCA-injected mice at 5 d. n=8. (E) MuRF1 and FBXO32 mRNA levels were measured in injured muscle of control and LCA-injected mice at 5 d. n=8. (F–I) Western blot analysis of protein turnover signaling pathway associated protein levels in C2C12 cells of control and 50 μM LCA treatment for 3 d in differentiation medium. n=6. Data are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; Student’s t test.
Figure 5. LCA promotes cell differentiation through TGR5 in C2C12 cells

(A,B) Western blot analysis of TGR5 receptor protein levels in injured muscle of control and LCA-injected mice at 5 d. *n=8. (C,D) Western blot analysis of TGR5 receptor protein levels in C2C12 cells of control and 50 μM LCA treatment for 3 d in differentiation medium. *n=6. (E,F) Western blot analysis of the TGR5 receptor in siCtrl, LCA and LCA + siTGR5 C2C12 cells at 3 d in differentiation medium. *n=6. (G,H) Western blot analysis of myogenic factor protein levels after 3 d in differentiation medium. *n=6. (I–L) Western blot analysis of protein turnover signaling pathway-associated protein levels at 3 d in differentiation medium. *n=6. Data are shown as the mean ± SEM. *P<0.05, **P<0.01; Student’s t test.
653x702mm (192 x 192 DPI)
688x625mm (192 x 192 DPI)
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<tr>
<th>Gene</th>
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<td>TGR5</td>
<td>F: TGCCTCCCTTCTCCACTTGAC&lt;br&gt;R: GCAAAGAACAGGGAGCTGAC</td>
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<tr>
<td>MyoD</td>
<td>F: GCTTCTATCGCCGCCCCTCC&lt;br&gt;R: CGCACATGCTCATCCTCACG</td>
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<td>MyoG</td>
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<td>MuRF1</td>
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<td>β-actin</td>
<td>F: GGTCATCTATTTGGCAACG&lt;br&gt;R: GAGGTCTTTACGGATGTAACG</td>
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*TGR5*, G proteins-coupled bile acid; *MyoD*, Myogenin differentiation; *MyoG*, Myogenic factor 4; *MuRF1*, muscle RING F-box; *FBXO32*, muscle atrophy F-box. F, forward; R, reverse.
Highlight

LCA promotes skeletal muscle regeneration through activating the TGR5 receptor and causing changes in downstream related genes. In this study, we find that LCA can activate the TGR5/AKT signaling pathway, inhibit protein degradation and promote protein synthesis to enhance the myogenic process and promote skeletal muscle regeneration.

- Treatment with LCA reduces the necrosis rate of injured skeletal muscle.
- Treatment with LCA promotes the proliferation and differentiation of injured skeletal muscle and C2C12 cells.
- Treatment with LCA affects the protein metabolism of injured skeletal muscle and C2C12 cells.
- Silencing the TGR5 reverses the expression effect of related proteins.