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RESEARCH ARTICLE

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Upregulation of circ-FBL promotes myogenic proliferation in myasthenia gravis by regulation of miR-133/PAX7

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Abstract

Myasthenia gravis (MG) is a disease involving neuromuscular transmission that causes fatigue of skeletal muscles and fluctuating weakness. It has been shown that impairment of myogenic differentiation and myofiber maturation may be the underlying cause of MG. In this study, we detected the abnormal expression of circular RNA (circRNA) using next-generation sequencing in patients with MG. We then investigated the regulatory mechanism and the relationship among circRNA, microRNA, and messenger RNA using quantitative reverse-transcription polymerase chain reaction, bioinformatics analysis, and luciferase report analysis. The expression of inflammatory cytokines and regulatory T lymphocytes was shown to be increased. Circ-FBL was significantly increased in MG patients. Bioinformatics and luciferase report analyses confirmed that miR-133 and PAX7 were the downstream targets of circ-FBL. Overexpression of circ-FBL promoted myoblast proliferation by regulation of miR-133/PAX7. Taken together, our study showed that upregulation of circ-FBL promoted myogenic proliferation in patients with MG by regulating miR-133/PAX7.

KEYWORDS

circ-FBL, miR-133, myasthenia gravis, myogenic differentiation, PAX7

1 | INTRODUCTION

Myasthenia gravis (MG), an autoimmune disorder, affects the neuromuscular junction (NMJ) and is characterized by circulating autoantibodies against post-synaptic membrane components. In approximately 85% of patients, the autoantibodies target the nicotinic acetylcholine receptor (AChR) (Conti-Fine et al., 2006; Sacks et al., 2018). The pathogenesis of MG is complicated. The ectopic expression of cytokines likely participates in the development of MG due to important roles in immune responses and inflammatory diseases (Binks et al., 2016; Gilhus, 2009). Interleukin-9 (IL-9) is a multifunctional cytokine that is involved in the immunopathologic changes and protective immunity due to specific disease settings and microenvironments (Yao et al., 2018). The IL-23/Th17 cell pathway is a potential target to diminish persistent thymic inflammation in patients with MG (Villegas et al., 2019). A previous study showed that upregulation of miR150-5p in MG patients is associated with reduced serum IL-17 levels and increased serum IL-10 levels (Ao et al., 2020).

Myogenesis is executed by muscle stem cells, that is, satellite cells (SCs). Muscle SCs are functionally impaired in patients with MG. Indeed,

Abbreviations: AChR, autoantibodies target the nicotinic acetylcholine receptor; EdU, 5-ethynyl-2′-deoxyuridine; IL-9, interleukin-9; MG, myasthenia gravis; NMJ, neuromuscular junction; SC, satellite cell.

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the autoimmune attack in patients with MG has been shown to have pathogenic effects on SCs and muscle regeneration (Attia et al., 2017). PAX7, an upstream regulator of myogenesis, has an important role in regulating SC self-renewal (Addicks et al., 2019; Buckingham & Relaix, 2015). Furthermore, Pax7 is essential for normal SC function in adult skeletal muscles (von Maltzahn et al., 2013).

It has been shown that abnormal expression of circRNA is associated with a number of diseases (Kristensen et al., 2019). The role of circRNA in patients with MG, however, has not been established. Thus, the purpose of the current study was to identify the role of circRNA in patients with MG.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

C57BL/6 mice, 4 weeks old and weighing 15–20 g (SLARC) were used in the present research. The Ethics Committee of Shanghai Pudong New Area Gongli Hospital approved the animal experiments (No. for the application: 2018007).

2.2 | Patients

We enrolled 35 MG patients and 11 volunteers without MG after obtaining informed consent at the Shanghai Pudong New Area Gongli Hospital from January 2019 to January 2020. The Board and Ethics Committee of Shanghai Pudong New Area Gongli Hospital granted permission for this project.

2.3 | Strand-specific next-generation RNA-Seq library construction

Total RNA was extracted from sera of patients with and without MG using TRIzol reagent (Invitrogen). Approximately 3 μg of total RNA was restricted from each sample using the VAHTS Total RNA-seq (H/M/R) Library Prep Kit from Illumina (Vazyme Biotech Co., Ltd.) to remove ribosomal RNA, while preserving other RNA classes, including noncoding RNAs and mRNAs. RNAs were treated with 40 U of RNase R (Invitrogen) at 37°C for 3 h, and purified with TRIzol. RNA-seq libraries were generated using a KAPA Stranded RNA-Seq Library Prep Kit (Roche), and subjected to deep sequencing using an Illumina HiSeq. 4000 (Aksomics, Inc.).

2.4 | Animals and induction of experimental autoimmune myasthenia gravis (EAMG)

EAMG was induced in the mice by injecting Torpedo AChR (30 μg/mouse) (Santa Cruz Biotechnology, Inc.), and the level of weakness was assessed by a blind evaluator according to the method described in a previous study (Shi et al., 2015). In general, mice with grade 2 weakness, which was defined as weakness at rest, were used in this study. EAMG and healthy mice were sacrificed following analysis. We collected 150 μl of blood from each mouse through the tail vein. After collection, blood samples were left to clot by leaving the sample undisturbed at room temperature. Then, we centrifuged samples at 2000*g* for 10 min in a refrigerated centrifuge to isolate the sera.

2.5 | Total RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Our team isolated total RNA from muscle tissues, cells, and sera with TRIzol reagent (Invitrogen). using standard techniques, then tested RNA sample purity and concentration by spectrophotometry. Absorbances at 230, 260, and 280 nm were determined with a NanoDrop ND-1000 (Thermo Fisher Scientific). Specifically, OD_{260}/OD_{280} ratios ranging from 1.8 to 2.1 and OD_{260}/OD_{230} ratios > 1.8 were considered acceptable.

2.6 | Cell culture

The mouse myoblasts (C2C12) and the 293T cell lines were purchased from American Type Culture Collection (ATCC). C2C12 cells were cultured in a growth medium (GM), supplemented with DMEM (Hyclone), 10% fetal bovine serum, and 1% penicillin-streptomycin (Gibco).

2.7 | Dual-luciferase reporter assays

Circ-FBL and PAX7 3′-UTR binding sites (circ-FBL-WT, circ-FBL-Mut, PAX7-3′-UTR-WT, and PAX7-3′-UTR-Mut) were inserted into the pGL3 promoter vector (Realgene) using the dual-luciferase reporter assay. Cells were dispensed into 24-well plates and transfected with 80 ng of plasmid, 50 nM miR-133 mimics, 5 ng Renilla luciferase vector pRL-SV40, and NC reagents with lipofectamine 2000 (Invitrogen). Cells were collected and measured 2 days following transfection using the dual-luciferase assay (Promega) following standard procedures. All experiments were repeated three times.

2.8 | 5-Ethynyl-2′-deoxyuridine (EdU) assay

We used the EdU assay kit (RiboBio) to study cell proliferation and DNA synthesis by seeding 10,000 cells into 96-well plates overnight. On Day 2 we added EdU solution (25 μM) to the plate and incubated cells for 1 day, utilized 4% formalin to fix cells at room temperature for 2 h, used 0.5% TritonX-100 to permeabilize cells for 10 min, and added Apollo reaction solution (200 μl) to stain EdU and 4′,6-diamidino-2-phenylindole (200 μl) to stain nuclei for 30 min. A microscope (Nikon) was used to determine cell proliferation and DNA synthesis, which were denoted by blue and red signals, respectively.

2.9 | Immunohistochemistry

Muscle tissue samples were immersed in 4% paraformaldehyde and embedded in paraffin. Tissue Sections (5-μm thick) were stained with Pax7 and laminin, and then examined with an Axiophot light microscope and imaged using a digital camera.

2.10 | Statistical analysis

The differences between groups were evaluated using paired/unpaired t-tests (two-tailed). Pearson's correlation test was used to determine correlations between the groups. Data are presented as the mean ± *SEM*, and p values < .05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism (GraphPad, Inc.).

3 | RESULTS

3.1 | Expression of inflammatory cytokines and the number of regulatory T lymphocytes were increased

MG is a neuromuscular disorder caused by anti-AChR autoantibodies that damage the neuromuscular signal transmission and influence skeletal muscle homeostasis. In the current study, the expression of inflammatory cytokines (TNF-α and IL-1β) in sera from MG patients was increased when compared with normal controls (Figure 1a,b). In addition, the regulatory T cell count was increased in MG patients (Figure 1c). In addition to immune abnormalities, SC activation and proliferation also play an important role in patients with MG. Pax7 is a SC marker, and RT-qPCR showed that serum Pax7 expression was increased in patients with MG (Figure 1d). We demonstrated that the number of Pax7-positive SCs (in red) localized between the basal lamina and sarcolemma was characterized by laminin staining (in green) and increased in the muscles of MG patients compared to controls (Figure 1e,f). Indeed, the number of SCs increased in mice and patients with MG.

3.2 | Circ-FBL was abnormally expressed in MG patients

Existing evidence confirms that abnormal circ-FBL expression is correlated with many diseases. Nevertheless, the function of circRNA in the pathogenesis of MG has not been established. Thus, in the current study, next-generation sequencing of circRNA revealed that many circRNAs are abnormally expressed (Figure 2a) in patients with MG. Among the circRNA, circ-Atad2, circ-Ldha, circ-Rnf2, circ-Ttc7b, circ-Myof, circ-Tra2b, circ-Ncor1, circ-Cspp1, circ-Smad4, and circ-FBL expression in MG patients were significantly increased. RT-qPCR detection further confirmed that among the upregulated circRNAs, circ-FBL (hsa_circ_0051032) was significantly increased in MG patients (Figure 2b).

3.3 | miR-133 and PAX7 are the downstream targets of circ-FBL

Bioinformatics analysis (http://starbase.sysu.edu.cn/) indicated that miR-133, miR-513a, miR-146, miR-370, miR-217, miR-144, miR-153, and miR-627 were downstream targets of circ-FBL. Then, the circ-FBL sequence was cloned into a luciferase reporter vector (WT-NC) and different microRNA (miRNA) mimics were transfected into 293T cells combined with WT-NC. The luciferase reporter analysis results indicated that miR-133 inhibited luciferase activity (Figure 3a), suggesting that miR-133 was a circ-FBL downstream target.

To further demonstrate the correlation between miR-133 and circ-FBL, mutated (MUT) and wild-type (WT) circ-FBL sequences were prepared that contained a miR-548 binding sequence into a luciferase reporter vector (Figure 3b). The reporter vector was subsequently transfected into 293T cells with or without a miR-133 mimic. Luciferase reporter results showed that miR-133 inhibited luciferase activity in WT-transfected cells, but not in MUT-transfected cells (Figure 3c), confirming that miR-133 is a circ-FBL target. RT-qPCR showed that miR-133 was increased in the sera of MG patients (Figure 3d).

Bioinformatical analysis validated that Pax7 was a miR-133 downstream target. To verify the correlation between Pax7 and miR-133, we created MUT or WT 3′-UTR-Pax7 sequences and integrated the miR-133 binding sequence into a luciferase reporter vector (Figure 3e). The reporter vector was transfected into 293T cells incorporated with or without miR-133 mimic. Luciferase reporter analysis illustrated that miR-133 inhibited luciferase activity in WT-transfected cells, but not in MUT-transfected cells (Figure 3f), indicating that Pax7 is a miR-133 target.

3.4 | Overexpression of circ-FBL promoted myoblast proliferation by regulating miR-133/PAX7

To characterize the regulatory correlations between circ-FBL, miR-133, and PAX7, myoblasts cells were transfected with circ-FBL, miR-133 mimic, or PAX7 silence vector (si-PAX7), either independently or by integration. RT-qPCR demonstrated that circ-FBL expression was remarkably increased following transfection with the circ-FBL overexpression vector. MiR-133 overexpression or PAX7 silencing did not reverse circ-FBL expression (Figure 4a). Circ-FBL overexpression decreased miR-133 expression in myoblasts. Using cells with miR-133 mimic promoted miR-133 expression, and PAX7 silence was not able to reverse miR-133 expression (Figure 4b). We also found that circ-FBL overexpression increased PAX7 expression in myoblasts, while miR-133 mimic treatment partially suppressed PAX7 expression. Transfecting a PAX7 silence vector decreased PAX7 expression (Figure 4c). In summary, the data showed that miR-133 and PAX7 are circ-FBL downstream targets and PAX7 is a miR-133 downstream target.

EdU (Figure 4d,e) assays showed that overexpression of circ-FBL promoted proliferation of myoblasts. Overexpression of miR-133 or PAX7 silencing suppressed the proliferation ability of myoblasts even after overexpression of circ-FBL.

4 | DISCUSSION

Separate microenvironments reflect multiple differentiation potentials involving mesenchymal stromal cells. For example, an inflammatory microenvironment suppresses myogenic differentiation capability regarding laryngeal mucosa mesenchymal stromal cells [LM-MSCs] (Yang et al., 2018). In the current study, we also found that inflammatory cytokines (TNF-α and IL-1β) in MG patients were increased. A number of inflammatory cytokines inhibit myogenic differentiation (Langen et al., 2001). The regulatory T lymphocyte count was also shown to be enriched in MG patients, which was consistent with a previous report (Schiaffino et al., 2017). T cell

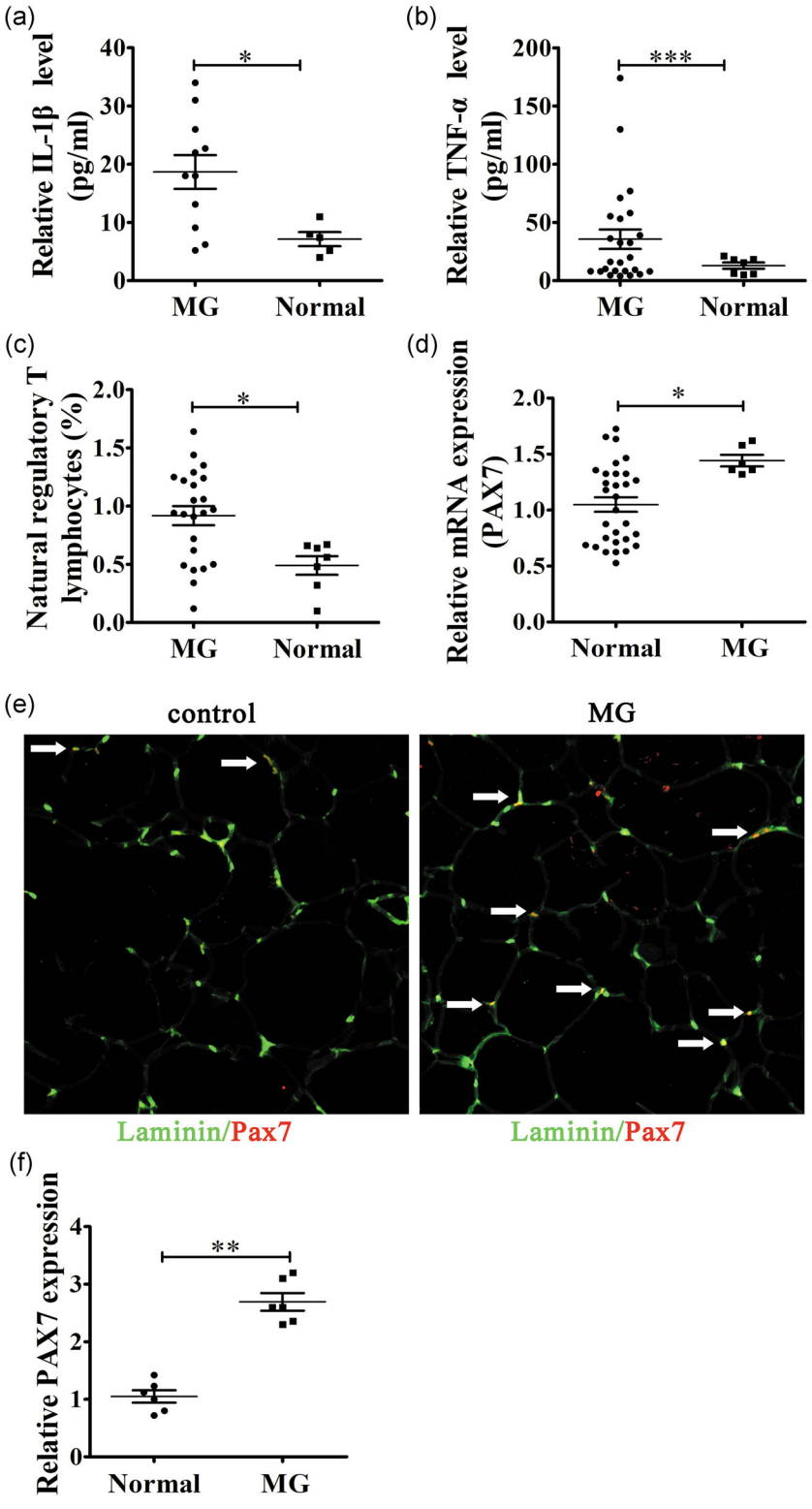

FIGURE 1 The expression of inflammatory cytokines and the number of regulatory T lymphocytes were increased. (a,b) ELISA showed the expression of inflammatory cytokines (IL-1β and TNF-α). Data are presented as the mean ± *SD*. $^{*}p < .05$, $^{***}p < .001$ versus normal. (c) The change in the number of regulatory T lymphocytes. Data are presented as the mean ± *SD*. $^{*}p < .05$ versus normal. (d) RT-qPCR showed the level of PAX7 mRNA expression. Data are presented as the mean ± *SD*. $^{*}p < .05$ versus normal. (e and f) Immunofluorescence showed the expression of Pax7 and laminin in mouse controls and MG skeletal muscles. White arrows show SCs, which are located between the basal lamina (yellow dotted line) and sarcolemma. Data are presented as the mean ± *SD*. $^{**}p < .001$ versus control. ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; MG, myasthenia gravis; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; SC, satellite cell; TNF-α, tumor necrosis factor-α

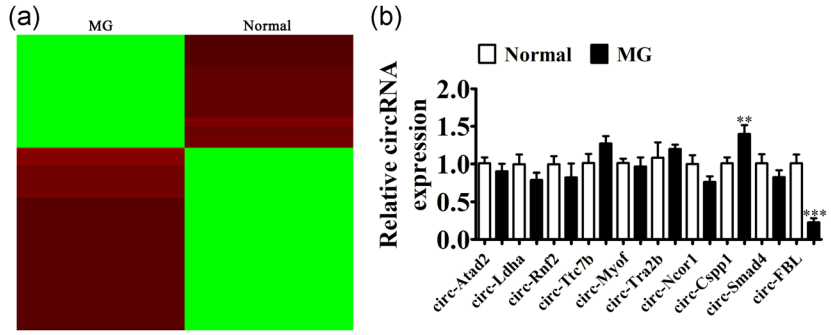

FIGURE 2 Circ-FBL was abnormally expressed in MG patients. (a) Heat map shows the differentially expressed circRNAs in patients with and without MG. (b) RT-qPCR showed the expression of six different circRNAs. Data are presented as the mean ± *SD*. $^{**}p < .01$, $^{***}p < .001$ versus normal. circRNA, circular RNA; MG, myasthenia gravis; RT-qPCR, quantitative reverse-transcription polymerase chain reaction

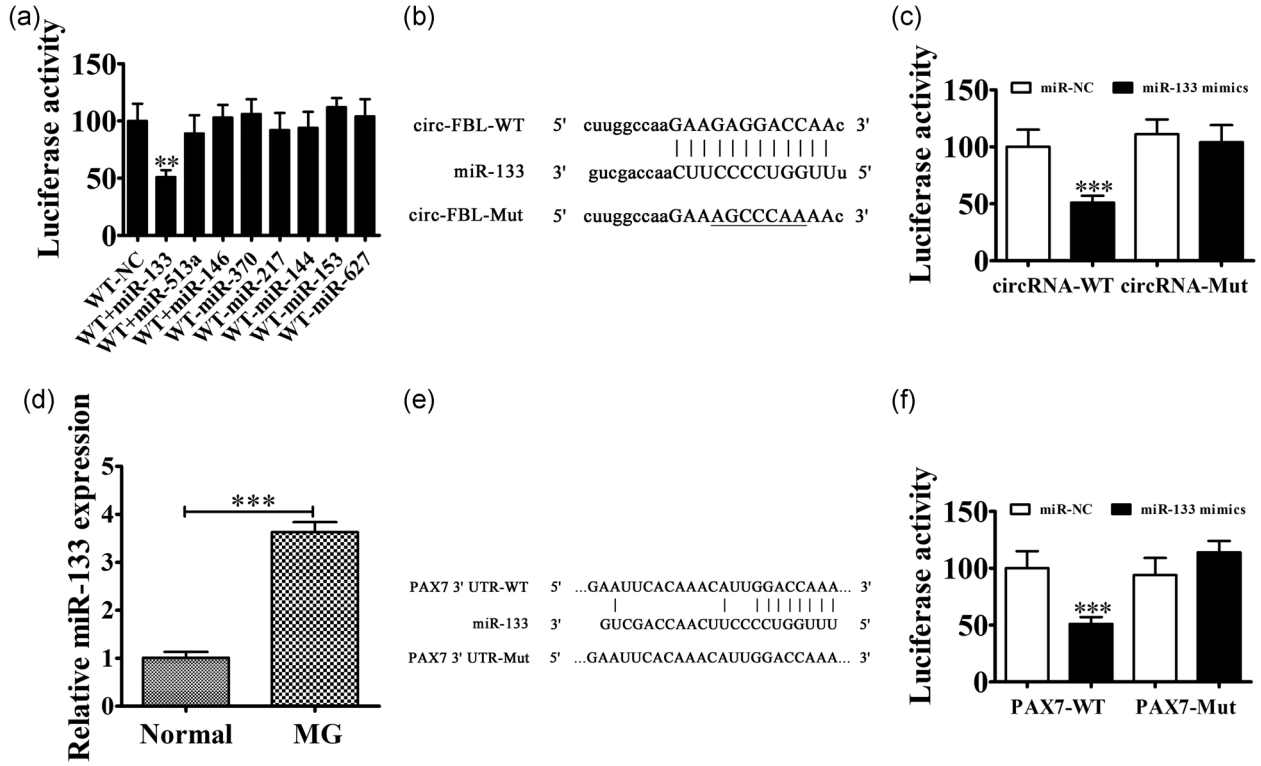

FIGURE 3 miR-133 and PAX7 were the downstream targets of circ-FBL. (a) Luciferase report analysis showed the putative binding ability of eight predicted miRNAs on circ-FBL. Data are presented as the mean ± *SD*. $^{**}p < .01$ versus NC. (b) RT-qPCR showed the relative expression of miR-133 in sera from patients with and without MG. Data are presented as the mean ± *SD*. $^{***}p < .001$. (c) The predicted binding sites of miR-133 in the circ-FBL. The mutated (Mut) version of circ-FBL is also shown. (d) Relative luciferase activity was determined 48 h after transfection with miR-133 mimic/normal control (NC) or with the circ-FBL wild-type/Mut in HEK293T cells. Data are presented as the mean ± *SD*; $^{***}p < .01$ versus NC. (e) The predicted binding sites of miR-519 within the 3′-UTR of PAX7. The mutated version of the 3′-UTR of PAX7 is also shown. (f) Relative luciferase activity was determined 48 h after transfection with the miR-133 mimic/normal control or with the 3′-UTR-PAX7 wild-type/Mut in HEK293T cells. Data are presented as the mean ± *SD*; $^{***}p < .01$ versus NC. 3′-UTR, 3′-untranslated region; MG, myasthenia gravis; miR-133, microRNA-133; miRNA, microRNA; Mut, mutated; NC, normal control; RT-qPCR, quantitative reverse-transcription polymerase chain reaction

recruitment regulates immune and inflammatory responses and regulates skeletal muscle precursor cell activities, which are essential for regeneration (Castiglioni et al., 2015).

Our investigation suggested that PAX7 expression is slightly increased in MG patients. Pax7 is necessary for normal SC function in adult skeletal muscle (von Maltzahn et al., 2013). Pax7 induction in muscle-derived SCs induces SC specificity by restricting alternate developmental programs. Pax7 is necessary for regulating SC expansion and differentiation in neonatal and adult myogenesis (Marg et al., 2019; Seale et al., 2000). Upregulation of Pax7 may

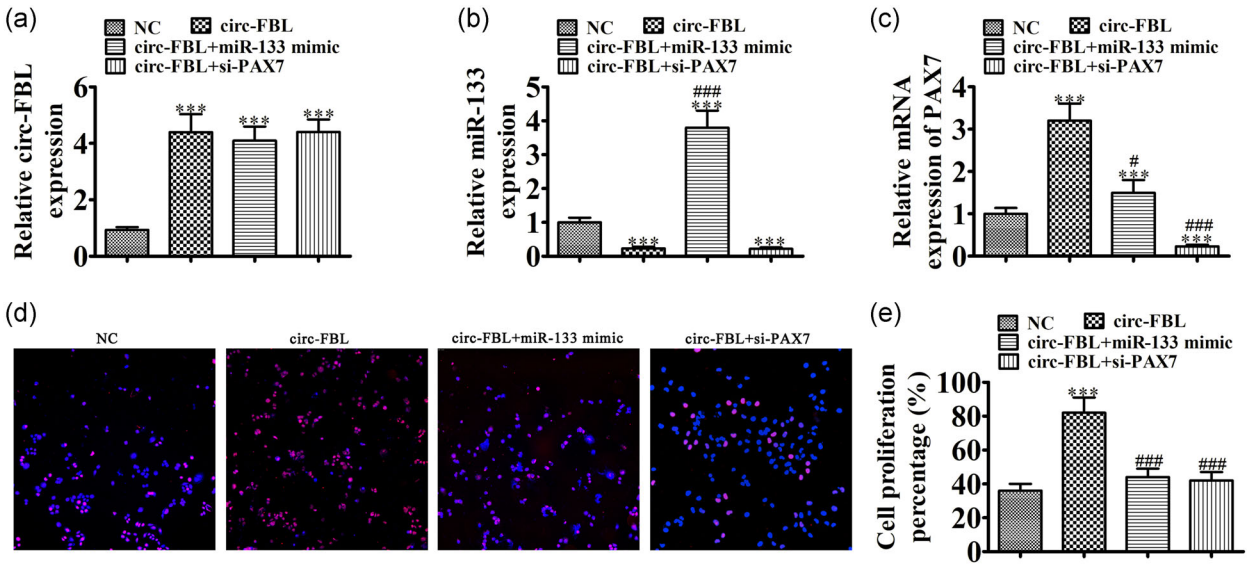

FIGURE 4 Overexpression of circ-FBL promoted myoblast proliferation by regulating miR-133/PAX7. (a–c) RT-qPCR showed the expression of circ-FBL (a), miR-133 (b), and PAX7 (c). Data are presented as the mean ± *SD*. $^{***}p < .001$ versus NC. $^{\#}p < .05$, $^{\#\#\#}p < .001$ versus circ-FBL. (d and e) Edu assay showed the proliferation of myoblasts after transfection with circ-FBL, miR-133 mimic, or PAX7 silence vector, either singly or in combination. Data are presented as the mean ± *SD*. $^{***}p < .001$ versus NC. $^{\#\#\#}p < .001$ versus circ-FBL.
Edu, 5-ethynyl-2′-deoxyuridine; miR-133, microRNA-133; RT-qPCR, quantitative reverse-transcription polymerase chain reaction

be a compensatory effect in patients with MG. Massive promotion of Pax7 expression may have a therapeutic effect on patients with MG.

A number of studies have discovered that abnormal circRNA expression is associated with many diseases (Chen & Yang, 2015; Cooper et al., 2018). Unlike other cellular RNAs, circular (circ) RNAs contain various noncoding (nc)RNAs without 5′ or 3′ ends. Due to this structure, circRNAs are not easily degraded by exonuclease RNase R (Chen & Yang, 2015). The role of circRNA in the pathogenesis of MG is still unknown. Thus, we used circRNA in next-generation sequencing and showed that circ-FBL expression increased in patients with MG. Currently, circRNAs mainly function by adsorbing miRNAs as miRNA response elements (MREs), as suggested by the competing endogenous RNA (ceRNA) hypothesis (Salmena et al., 2011; Tay et al., 2014). In the current study, bioinformatics and luciferase reporting experiments confirmed that miR-133 and PAX7 are the downstream targets of circ-FBL. Overexpression of circ-FBL promoted PAX7 expression by absorbing miR-133. RT-qPCR showed that miR-133 expression was increased in MG patients. A previous study demonstrated that miR-133 expression suppresses cardiac remodeling (Li et al., 2018). MiR-133 upregulated by circ-FBL promoted myoblast proliferation.

5 | CONCLUSIONS

Although previous studies have focused on circRNA in patients with cancer, cardiovascular, and cerebrovascular diseases, we constructed a circRNA–miRNA–mRNA regulatory network that showed that upregulation of circ-FBL promoted myogenic differentiation in patients with MG by regulation of miR-133/PAX7. It is essential to combine basic experiments with clinical data to further investigate circRNA and identify new molecular markers related to the prognosis, prediction, and therapeutic targets for clinical patients.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

All data included in the manuscript are available.

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