Forkhead Box M1 Transcriptionally Regulates the Expression of Long Noncoding RNAs Snhg8 and Gm26917 to Promote Proliferation and Survival of Muscle Satellite Cells

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ABSTRACT

Multiple functions have been proposed for transcription factor FoxM1, including the regulation of cell proliferation, differentiation, senescence, apoptosis, and tissue homeostasis. However, the role of FoxM1 in muscle satellite cells (SCs) remains unclear. In the present study, we demonstrated that FoxM1 was essential for the proliferation and survival of SCs. Crucially, we found that long noncoding RNAs (lncRNAs) Snhg8 and Gm26917 significantly regulated the proliferation and apoptosis of SCs, respectively, and these IncRNAs were directly regulated by FoxM1 in SCs. Mechanistically, Snhg8 sustained SCs proliferation by promoting the transcription of ribosomal proteins, while Gm26917 acted as a competing endogenous RNA for microRNA-29b, which accelerated apoptosis of SCs. In mice, conditional knockout of FoxM1 in skeletal muscle resulted in decreased proliferation and increased apoptosis of SCs. Thus, our studies revealed a previously unrecognized role of FoxM1 in SCs and uncovered two lncRNAs, Snhg8 and Gm26917, which function as novel targets of FoxM1 in the regulation of SC proliferation and survival.

SIGNIFICANCE STATEMENT

Precise regulation of muscle satellite cells (SCs) quiescence, proliferation, and differentiation is crucial for muscle homeostasis. This article reports an important role for transcription factor FoxM1 in the maintenance of SCs. Derelegation of FoxM1 expression led to impaired proliferation and survival of SCs. This study further suggested that the FoxM1-Snhg8 and FoxM1-Gm26917 axis sustains the proliferation and survival of SCs in a tissue-specific manner. The study uncovered long noncoding RNA genes that are regulated by FoxM1, and provides insights into the molecular network of SCs regulation.

INTRODUCTION

Muscle satellite cells (SCs) are a population of muscle stem cells located between the basal lamina and the plasma membrane of muscle fibers [1]. They play essential roles in homeostasis and regeneration of skeletal muscles, and are characterized by their ability for quiescence, self-renewal, and differentiation [2]. In response to stimuli (e.g., damage or exercise-induced contraction), SCs break quiescence, undergo proliferative expansion, and terminally differentiate to form myotubes to replace the damaged myofibers; while a subset of activated SCs exit the cell cycle to replenish the muscle stem cells pool [3]. Numerous genes are involved in the precise control of quiescence, proliferation, and differentiation of SCs. However, the underlying mechanisms remain unclear.

The forkhead box M1 (FoxM1), a member of the Fox transcription factor family, plays important roles in the regulation of cell proliferation and apoptosis [4]. FoxM1 transcriptionally activates important pro-proliferative genes (including those encoding Cks1, Cdc25b, Cyclin A2, and Cyclin B) and promotes cell cycle progression [5]. FoxM1 also regulates cell apoptosis and cellular survival via its downstream target genes, including those encoding Bcl2, p27, and JNK [6]. Several studies have uncovered the diverse functions of FoxM1 in stem cells, which are mainly related to self-renewal, cell-cycle progression, and survival. Studies have demonstrated that FoxM1 is essential for the maintenance of cell proliferation and...
of SCs in vivo and in vitro. Global gene expression analysis ever, little is known about the novel lncRNAs regulated by downstream target genes of FoxM1 have been reported. How-ever, differentiation and muscle regeneration [21]. Currently, many certain lncRNAs significantly regulate the proliferation and differ-entiation of SCs, such as lnc-Malat1, Inc-000961, Inc-YY1, and Inc-31 [16–19]. In terms of their transcriptional profile, the pattern of lncRNAs expression tends to developmental stage and cell type-specific [20]. Transcriptional regulation of lncRNAs expression partly contributes to this phenomenon. For instance, IncRNA-Dum is transcriptionally induced by myo-genic transcription factor (MyoD) binding and is dynamically regulated during myogenesis, which promotes myoblast differ-entiation and muscle regeneration [21]. Currently, many downstream target genes of FoxM1 have been reported. How-ever, little is known about the novel lncRNAs regulated by FoxM1, especially in SCs.

In the present study, we investigated the function of FoxM1 in SCs using the C2C12 cell line and mouse models with conditional deficiency of FoxM1. We found that loss of FoxM1 impaired proliferation and accelerated the apoptosis of SCs in vivo and in vitro. Global gene expression analysis identified 1046 mRNAs and 49 lncRNAs that showed differential expression between FoxM1-knockdown C2C12 cells and control cells. Importantly, we identified that the lncRNAs Snhg8 (small nucleolar RNA host gene 8) and Gm26917 (predicted gene, 26917) were direct targets of FoxM1. Notably, downregulation of Snhg8 inhibited the transcription of ribosomal protein genes, thus impeding SCs proliferation. Over-expression of Snhg8 in FoxM1-knockout SCs rescued the impaired proliferation. In addition, Gm26917 was required for SCs survival via interaction with microRNA (miR)-29b. Over-expression of Gm26917 in FoxM1-knockout SCs rescued the survival of SCs. Taken together, our data provided evidence that FoxM1 is a critical regulator of proliferation and survival of SCs, mediated in part by lncRNAs Snhg8 and Gm26917.

MATERIALS AND METHODS

Animals

The mice used had a C57BL/6J background. For conditional deletion of FoxM1 in vivo, we established a mouse line in which the FoxM1 was deleted by Cre recombinase, driven by a Pax7 promoter. The FoxM1<sup>-/-</sup> mouse was crossed with Pax7-Cre mouse to generate FoxM1<sup>-/-</sup>-Pax7-Cre conditional knockout mice (FoxM1-cKO). For muscle injury, a total of 70 μl of 1.2% BaCl<sub>2</sub> (wt/vol H<sub>2</sub>O) were injected into the tibialis anterior (TA) muscles and the injured muscles were harvested at 60 hours after injection [22]. All data were obtained from mice at 8–15 weeks of age. All the mice experiments were approved by the Animal Committee of the Institute of Zoology, Third Military Medical University.

Isolation of Single Myofibers and SCs

To detect the expression pattern of FoxM1 in SCs in vivo, single myofibers were isolated from extensor digitorum longus (EDL) muscles and digested in Dulbecco’s modified Eagle’s medium (DME(M) (catalogue number: SH30022.01, Hyclone, GE, USA) with 0.2% collagenase (Sigma-Aldrich, USA) at 37°C for 90 minutes. Fibers were liberated by trituration in DME medium with Pasteur pipettes. The fibers were then fixed for immunofluorescence. In addition, fluorescence activated cell sorting (FACS) was used to isolate SCs (quiescent) and non-SCs. Briefly, TA muscles of mice were digested with 0.2% collagenase for 90 minutes and then with 0.2% dispase (Sigma-Aldrich) for 30 minutes. The cell suspension was filtered through a 70-μm nylon filter (Falcon, Corning, USA) and mononuclear cells were collected and subjected to FACS (BD FACSAriaII, USA) using immuno-nostaining with biotinylated antibodies recognizing the following proteins: CD45, CD11b, CD31, and Sca1; as well as those recognize-nig streptavidin-APC-Cy7, CD34-Alexa Fluor 647, and Integrin α7-FITC (all eBioscience, Thermo Fisher, USA) [23, 24]. SCs are defined as CD45<sup>-</sup>Sca1<sup>-</sup>CD11b<sup>-</sup>CD31<sup>-</sup>CD34<sup>-</sup>α7-integrin<sup>+</sup>, while non-SCs are defined as CD45<sup>-</sup>Sca1<sup>-</sup>CD11b<sup>-</sup> CD31<sup>-</sup>CD34<sup>-</sup>α7-integrin<sup>−</sup>. Isolated SCs and non-SCs were immedia-tely lysed for RNA extraction.

To culture SCs in vitro and avoid harming the SCs generated by FACS, we obtained SCs using single myofiber cultures [25, 26]. Briefly, single fibers were placed in Matrigel-coated dishes (BD Bioscience, USA) in fiber medium consisting of DMEM (Hyclone) with 20% fetal bovine serum (Hyclone), 1% penicillin/ streptomycin (Hyclone), and 1% Chick embryo extract (US bio-logical, USA) at 37°C with 5% CO<sub>2</sub>. SCs migrated off the myofibers in 3–4 days. To analyze the growth of SCs, isolated SCs were cultured in fiber medium. For differentiation, SCs were cultured in DMEM containing 2% horse serum (Gibco, Thermo Fisher, USA) on Matrigel.

RNA-Sequence (RNA-Seq) and Bioinformatics Analysis

To identify the potential pathways regulated by FoxM1, C2C12 cells were used for RNA-Seq analysis. RNA was extracted from FoxM1-knockdown C2C12 cells and control cells using TRIzol (Invitrogen, Thermo Fisher, USA). Libraries were constructed after rRNA elimination and sequenced on the Illumina HiSeq 2000 platform as 150-bp pair-ended reads. Reads were aligned using Bowtie v0.12.9. Fragments per kilobase of transcript per million mapped reads (FPKM) estimation was performed using Cufflinks v2.1.1. Aligned reads were counted using HTSeq, and differential expression analysis was performed using DESeq2. Differentially expressed genes and lncRNAs were selected using a cutoff of a <i>p</i> value less than .05 (false discovery rate-adjusted for multiple testing). For functional profiling of changes in mRNAs by RNA-Seq, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using the web tool at The Database for
Rapidly upon Their Differentiation

In muscle tissues, SCs are located on the surface of myofibers and maintain a quiescent state with Pax7 expression. Upon muscle injury, they are activated and proliferate to form fibers (Fig. 1B). Furthermore, we demonstrated that FoxM1 was highly expressed in Pax7-positive, quiescent SCs on isolated myofibers from the EDL muscles from C57BL/6J wild-type mice, with or without BaCl2-induced skeletal muscles injury [22]. The immunofluorescence results showed that FoxM1 was highly expressed in Pax7-positive, quiescent (noninjured condition) or activated (injured condition) SCs, while little or no FoxM1 was found in Pax7-negative cells (Fig. 1A). FACS purified cells (CD45<sup>-</sup> Sca1<sup>+</sup> CD11b<sup>+</sup> CD31<sup>-</sup> CD34<sup>+</sup> α7-integrin<sup>+</sup>) have been previously described as SCs [24]. We sorted CD45<sup>-</sup> Sca1<sup>+</sup> CD11b<sup>+</sup> CD31<sup>-</sup> CD34<sup>-</sup> α7-integrin<sup>-</sup> cells, and found that FoxM1 was highly expressed in these cells compared with CD45<sup>-</sup> Sca1<sup>-</sup> CD11b<sup>-</sup> CD31<sup>-</sup> CD34<sup>+</sup> α7-integrin<sup>-</sup> cells (non-SCs). In addition, Pax7 showed similar expression pattern with FoxM1 (Fig. 1B). Furthermore, we demonstrated that the expression of FoxM1 decreased rapidly upon SCs differentiation in vitro (Fig. 1C, 1D).

C2C12 cells, a murine myoblast cell line, are a widely investigated model of myogenesis [18]. Initially, C2C12 cells were growth arrested in G0 (quiescent state) by culturing in semi-solid medium [27], became proliferative in growth medium, and differentiated in differentiation medium. The bromodeoxyuridine (BrdU) incorporation assay confirmed the cell-cycle kinetics of C2C12 in different states (Fig. 1E). Accordingly, quiescent C2C12 expressed the transcription factor Pax7 and when activated, coexpressed Pax7 with MyoD; while differentiated cells lost Pax7 expression and showed myogenin and integrin 7α expression (Fig. 1G) [28]. Notably, we found that FoxM1 was highly expressed in quiescent and activated C2C12, but was lost rapidly upon C2C12 differentiation (Fig. 1F, 1G). This result suggested an important role for FoxM1 in muscle stem cells.

Loss of FoxM1 Impaired Proliferation and Accelerated Apoptosis of Both SCs and C2C12 Cells

Given that FoxM1 was highly expressed in quiescent SCs and upregulated in activated SCs, we reasoned that FoxM1 might regulate the proliferation and survival of SCs. To address the role of FoxM1 in SCs, we used FoxM1-knockdown C2C12 cells using short hairpin RNAs (shRNAs) (Supporting Information Fig. S1D, S1E). The BrdU incorporation assay indicated that independent shRNAs reproducibly prevented cells entering S phase. While approximately 26% of shControl cells incorporated BrdU<sup>+</sup>, approximately 15% of the shFoxM1 cells were BrdU<sup>-</sup> (Fig. 2A, 2B). Notably, knockdown of FoxM1 had no significance on the cell-cycle kinetics of quiescent C2C12 cells (Supporting Information Fig. S1H). The observations were also supported by immunofluorescence and CCK8 experiments (Supporting Information Figs. S1I, S2C). To further investigate the fate of FoxM1-knockdown cells, apoptosis was measured by flow cytometry. The percentage of early apoptosis ( Annexin V<sup>-</sup>/PI<sup>-</sup> ) shFoxM1 cells was 2–3 fold higher than control C2C12 cells, both in the quiescent and proliferative states (Fig. 2D–2F). Collectively, the results suggested that FoxM1 regulates cell proliferation and survival of C2C12 cells in a context-dependent manner.

To investigate the function of FoxM1 in SCs, we crossed mice with loxP-flanked FoxM1 alleles (FoxM1<sup>fl/fl</sup>) with Pax7-Cre mice, in which a transgene encoding Cre recombinase is expressed in SCs under the control of the Pax7 promoter [25] to generate control FoxM1<sup>fl/fl</sup> mice and FoxM1<sup>fl/fl</sup>, Pax7-Cre (called “FoxM1-cKO mice” here). We confirmed the high efficiency of FoxM1 deficiency in SCs at the genomic DNA level (Supporting Information Fig. S1A), mRNA level (Supporting Information Fig. S1B), and protein level (Supporting Information Fig. S1C). We analyzed the expansion and survival of SCs in these mice at 8 weeks of age in vitro. FoxM1-cKO SCs showed less BrdU incorporation and reduced expansion in culture (Fig. 2G, 2H, Supporting Information Fig. S1J). In addition, flow cytometry revealed that knockout of FoxM1 strikingly accelerated the early apoptosis of SCs (Fig. 2I, 2J). Thus, our data indicated that deletion of FoxM1 impaired the expansion and survival of SCs.

Genes and Molecular Pathways Dysregulated in FoxM1-Deficient C2C12 Cells

To identify FoxM1-dependent genes and the molecular pathways involved in the regulation of SCs function, global gene expression profiles of FoxM1-knockdown C2C12 cells (referred to as shFoxM1 cells) and their corresponding control cells

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FoxM1 Directly Regulated the Transcription of Snhg8 and Gm26917

To determine whether FoxM1 regulates Snhg8 and Gm26917 expression through direct transcriptional activation, we searched for the consensus FoxM1-binding site (C/T)AAA(C/T)AA in the proximal promoter regions of Snhg8 and Gm26917. Gm27917 was the only detected lncRNA that was not in quiescent cells. Although the function of Snhg8 has not been clarified, an integrative analysis of lncRNAs and mRNA expression suggested that Snhg8 might target the ribosome pathway [29]. Consistently, we identified significant changes in the ribosome pathway within activated FoxM1-knockdown cells (Fig. 3D), which implied a crucial role of Snhg8 in SCs. Thus, these FoxM1 deficiency-mediated transcriptional changes indicated that knockdown of FoxM1 perturbed multiple mRNA pathways, as well as novel IncRNA pathways.

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The chromatin immunoprecipitation-PCR (Chip-PCR) results indicated that FoxM1 bound the P5 region of the Gm26917 promoter in both quiescent and activated C2C12 cells. Intriguingly, FoxM1 binding was only detected in the P2 region of the Snhg8 promoter in activated C2C12 cells, but not in quiescent cells (Fig. 4B). Moreover, Chip-qPCR demonstrated that FoxM1 directly bound to the P2 and P5 regions of Snhg8 and Gm26917 in activated C2C12 cells, respectively (Fig. 4C, 4D). Next, we performed dual-luciferase reporter assays using wild-type Snhg8 and Gm26917 promoters or promoters with mutations in the predicted FoxM1 binding sites. The luciferase activity of a construct containing the wild-type binding sites showed obvious increases with FoxM1 expression; however, the construct containing the mutated binding sites did not (Fig. 4E, 4F). Thus, these data demonstrated that FoxM1 directly binds to the promoters of Snhg8 and Gm26917.

To confirm the regulatory effect of FoxM1 on Snhg8 and Gm26917, we tested the correlation expression of FoxM1 and the lncRNAs. The qPCR results showed that knockdown of FoxM1 resulted in significantly downregulation of Snhg8 and Gm26917, except for the expression of Snhg8 in quiescent C2C12 cells (Fig. 4G–4J). These differences might be attributed to the selective regulation of FoxM1 on Snhg8 (as reflected by the Chip-PCR results). A similar effect was also found in FoxM1-cKO SCs (Fig. 4O, 4P). Moreover, the overexpression of FoxM1 promoted the expression of both Snhg8 and Gm26917 in activated C2C12 cells (Supporting Information Fig. S1F, S1G, Fig. 4K, 4L). Taken together, these data suggested that FoxM1 directly regulates the transcription of Snhg8 and Gm26917.
Knockdown of Snhg8 Reduced the Proliferation, While Knockdown of Gm26917 Impaired the Survival, of C2C12 Cells and SCs

To determine the functions of Snhg8 and Gm26917 in the proliferation and survival of SCs, we detected the expression patterns of the lncRNAs and found that Snhg8 was upregulated in activated C2C12 cells, but Gm26917 was not (Fig. 4M, 4N). Next, we used shRNAs to knockdown the expression of Snhg8 or Gm26917 in C2C12 cells and primary SCs, and confirmed the interference efficiency (Supporting Information Fig. S2A–S2D). We observed reduced proliferation (percentage of BrdU<sup>+</sup> cells) in both C2C12 cells and SCs when Snhg8 expression was knocked down by independent shRNAs, while knockdown of Gm26917 had no significant effect on proliferation (Fig. 5A–5C). Consistently, the immunofluorescence and CCK8 experiments demonstrated that Snhg8 knockdown impaired the proliferation of C2C12 and SCs (Supporting Information Fig. S3A–S3C). We then tested the effect of knockdown of Snhg8 and Gm26917 on apoptosis of C2C12 and SCs. Intriguingly, we found that knockdown of Gm26917 obviously accelerated apoptosis of both C2C12 and SCs; however, down-regulation of Snhg8 had no notable impact on cellular apoptosis (Fig. 5D–5F). Thus, these data suggested that Snhg8 and Gm26917 are necessary for SC proliferation and survival, respectively.

Snhg8 Sustained SCs Proliferation by Promoting Ribosome Protein Gene Transcription

It has been reported that Snhg8 might target the ribosome pathway [29], and we found that almost all ribosome proteins showed decreased expression in FoxM1-knockdown C2C12 cells (Fig. 6A). In addition, we selected several ribosome proteins (Rps10, Rps16, Rps27, Rpl10a, Rpl26, and Rpl36) and demonstrated their universal downregulation in FoxM1-cKO SCs (Fig. 6B). Thus, we hypothesized that FoxM1 regulates ribosome protein gene transcription via Snhg8. We found that independent shRNAs for Snhg8 reproducibly inhibited the expression of the above ribosome proteins, while overexpression of Snhg8 promoted the transcription of these genes in C2C12 cells (Fig. 6C, 6D). These data suggested a FoxM1-Snhg8-ribosome protein gene transcription axis in SCs. Ribosomes act as centers of protein synthesis, and comprise four noncoding RNAs and approximately 80 distinct ribosomal proteins [32]. The ribosome determines the capacity for protein production; therefore, ribosome biogenesis is the limiting step for cell proliferation [33]. To determine whether Snhg8 mediated the inhibited proliferation of FoxM1-deficient cells, we assessed the effect of overexpression of Snhg8 on the proliferation of FoxM1-knockdown C2C12 cells. Notably, the frequency of BrdU<sup>+</sup> cells was obviously greater among FoxM1-knockdown cells transfected with pCDH-Snhg8 than in cells

Figure 3. mRNAs and IncRNAs were dysregulated in FoxM1-knockdown C2C12 cells. (A, B): Heatmaps showing the top 100 differentially expressed protein coding genes (A) and all differentially expressed IncRNA genes (B) between FoxM1-knockdown (shFoxM1) and control (shControl) C2C12 cells in quiescent (SM) and proliferative (GM) states (p < .05). Putative IncRNA genes that are important for proliferation and survival of C2C12 cells are indicated. Three or four independent samples of shControl and shFoxM1 C2C12 were studied. (C, D): KEGG pathway analysis of dysregulated protein coding genes in FoxM1-knockdown C2C12 cells in quiescent (SM) and proliferative (GM) states. (E): Dysregulated protein coding genes directly involved in cell division (upper panel) in FoxM1-knockdown C2C12 cells (proliferative state). The lower panel shows dysregulated protein coding genes directly involved in apoptosis in FoxM1-knockdown C2C12 cells (quiescent or proliferative state). Abbreviations: FoxM1, forkhead box M1; GM, growth medium; IncRNAs, long noncoding RNAs; SM, semi-solid medium.
transfected with the empty vector (Fig. 6E, 6F). Consistently, the CCK8 results also demonstrated that overexpression of Snhg8 partly recovered the proliferative capacity of C2C12 cells (Supporting Information Fig. S3D). In addition, we also found that overexpression of Snhg8 partly recovered the proliferation of FoxM1-knockout SCs, while knockdown of Snhg8 or Gm26917 had no obvious additive effects on proliferation of FoxM1-knockout SCs (Fig. 6G, Supporting Information Fig. S3E, S3F). Taken together, these data proved that the FoxM1-Snhg8-ribosome protein gene regulation axis plays an important role in SC proliferation.

**Gm26917 Is Necessary for SC Survival via Interaction with miR-29b**

Currently, the function of Gm26917 has not been reported. Thus, we analyzed the potential interaction of Gm26917 with miR-29b. Figure 4. FoxM1 directly regulated the transcription of long noncoding RNAs Snhg8 and Gm26917. (A): Schematic diagram of the upstream promoter regions of the mouse Snhg8 and Gm26917 genes. Predicted FoxM1 binding sites are shown: site 1 (−1,857 to −1,852), site 2 (−1,307 to −1,297), and site 3 (−983 to −978) in the Snhg8 promoter region; and site 4 (−1,519 to −1,514), site 5 (−589 to −584), and site 6 (−245 to −240) in the Gm26917 promoter region. The amplified regions in the ChIP assay are indicated as P1, P2, P3, and NC1 for Snhg8; and P4, P5, P6, and NC2 for Gm26917. The NC1 and NC2 regions are located 10 kb upstream of the transcriptional start site and served as negative controls. (B): Endogenous binding of FoxM1 to the promoter regions of Snhg8 and Gm26917, as determined by a ChIP assay in C2C12 cells in quiescent (SM) and proliferative (GM) states. IgG was used as a negative control. (C, D): qPCR analysis of the binding of FoxM1 to the P2 region of Snhg8 and the P5 region of Gm26917 in proliferative C2C12 cells; IgG served as a negative control (n = 3). (E): Schematic diagram of luciferase constructs with wild-type (WT) or mutated (Mut) promoters of Snhg8 (P5) and Gm26917 (P6). (F): Luciferase activity in 293T cells transfected with a luciferase reporter vector with the WT or Mut promoter of Snhg8 (left panel), or the promoter of Gm26917 (right panel), as well as pCDH and pCDH-FoxM1. Results are presented relative to those of cells transfected with the luciferase construct and empty vector (pCDH), n = 3. (G–J): qPCR analysis of Snhg8 (G, H) and Gm26917 (I, J) in FoxM1-knockdown C2C12 cells (cultured in SM or GM), n = 3. (K, L): qPCR analysis of Snhg8 (K) and Gm26917 (L) in C2C12 cells (proliferative state) with the expression of FoxM1 (pCDH-FoxM1) or control vector (pCDH), n = 4. (M, N): qPCR analysis of Snhg8 (M) and Gm26917 (N) in C2C12 cells cultured in SM or GM, n = 3. (O, P): qPCR analysis of Snhg8 (I) and Gm26917 (J) in FoxM1 knockout SCs (proliferative state), n = 4. *, p < .05; **, p < .01. The results are expressed as means ± SD. Abbreviations: ChIP, chromatin immunoprecipitation; FoxM1, forkhead box M1; GM, growth medium; qPCR, quantitative polymerase chain reaction; SM, semi-solid medium.

transfected with the empty vector (Fig. 6E, 6F). Consistently, the CCK8 results also demonstrated that overexpression of Snhg8 partly recovered the proliferative capacity of C2C12 cells (Supporting Information Fig. S3D). In addition, we also found that overexpression of Snhg8 partly recovered the proliferation of FoxM1-knockout SCs, while knockdown of Snhg8 or Gm26917 had no obvious additive effects on proliferation of FoxM1-knockout SCs (Fig. 6G, Supporting Information Fig. S3E, S3F). Taken together, these data proved that the FoxM1-Snhg8-ribosome protein gene regulation axis plays an important role in SC proliferation.

**Gm26917 Is Necessary for SC Survival via Interaction with miR-29b**

Currently, the function of Gm26917 has not been reported. Thus, we analyzed the potential interaction of Gm26917 with
mRNA or DNA, but no putative targets with high probability were found. By contrast, we found that several miRNAs have potential matching fragments with Gm26917, including miR-425, miR-486, miR-28-5p, miR-29b, miR-100, miR-125b, and miR-320. We detected the expression patterns of the above microRNAs and found that miR-29b showed strikingly higher expression compared with the other miRNAs in C2C12 cells (Fig. 7A). Notably, we found that knockdown of FoxM1 significantly promoted the expression of miR-29b in C2C12 cells, as well as in FoxM1-cKO SCs (Supporting Information Fig. S2G, Fig. 7B). Moreover, overexpression of FoxM1 reduced the expression of miR-29b in C2C12 cells (Supporting Information Fig. S2H). These data suggested that miR-29b is a potential target of FoxM1.

MiR-29b plays an important role in muscle atrophy and aging-induced sarcopenia by targeting IGF1 and PI3K [34, 35]. In the present study, we also found that the expression of IGF1 was significantly decreased in FoxM1-deficient C2C12 cells (quiescent and activated states) and in FoxM1-cKO SCs (data from RNA-Seq and Supporting Information Fig. S2I). To investigate whether abnormal expression of miR-29b affected the apoptosis of SCs, we overexpressed miR-29b in C2C12 cells and SCs. The overexpression of miR-29b resulted in increased percentages of late apoptosis in C2C12 cells and SCs (Supporting Information Fig. S2J, Fig. 7C, 7D). The results indicated that the FoxM1-miR-29b axis was essential for survival of SCs. To determine whether FoxM1 regulates miR-29b via Gm26917, we interfered with the expression of Gm26917 and found that knockdown of Gm26917 promoted the expression of miR-29b (Fig. 7E). Gm26917 has a site that potentially matches miR-29b (Fig. 7F); therefore, we overexpressed wild-type Gm26917 or mutant Gm26917 (Supporting Information Fig. S2F). The qPCR results revealed that overexpression of Gm26917 inhibited the expression of miR-29b; however, mutant Gm26917 had no obvious effect (Fig. 7F). Moreover, the overexpression of Gm26917 significantly protected FoxM1-knockdown C2C12 cells against apoptosis, but overexpression of the mutant Gm26917 did not (Fig. 7G, 7H). In addition, we found that overexpression of Gm26917 obviously reduced the apoptosis of FoxM1-knockout SCs, while knockdown of Snhg8 or Gm26917 had no obvious additive effects on apoptosis of FoxM1-knockout SCs (Fig. 7I, Supporting Information Fig. S3G). Ultimately, these results demonstrated that the FoxM1-Gm26917-miR-29b axis was necessary for SC survival.

**DISCUSSION**

In this study, we discovered a previously unrecognized role for FoxM1 in the maintenance of SCs. Using the C2C12 cell
line and mouse models with conditional deletion of FoxM1, we demonstrated that FoxM1 was essential for the proliferation and survival of SCs in vivo and in vitro. Furthermore, we identified that FoxM1-Snhg8 and FoxM1-Gm26917 axes mediate the function of FoxM1 in regulating SC proliferation and survival, respectively.

FoxM1 serves as an important regulator for proliferation and cellular survival, and plays crucial roles during embryonic and fetal development, as well as in adult tissue homeostasis and repair [4]. Increasing evidence suggests the vital function of FoxM1 in stem cells. FoxM1 is essential for self-renewal and generation of induced pluripotent stem cells [36]. In particular, FoxM1 knockout embryos displayed a decreased number of hepatoblasts, cardiomyocytes, and lung mesenchymal cells [13]. Deletion of FoxM1 disturbed the quiescence and maintenance of mouse hematopoietic stem cells in vivo [8]. FoxM1 also regulates the self-renewal of glioma-initiating cells [37]. However, the role of FoxM1 in muscle SCs has not yet been characterized. Our findings provided evidence that FoxM1 is ubiquitously expressed in both quiescent and activated SCs, and decreases rapidly upon SC differentiation. We observed that loss of FoxM1 impaired proliferation and accelerated apoptosis of SCs, which suggested that FoxM1 intrinsically regulates the survival and proliferation of SCs. Our findings raised the possibility that the role of FoxM1 in tissue homeostasis and regeneration might be mediated in part by its impact on relevant tissue-specific stem cells.

Studies have shown that over 220 genes are regulated by FoxM1 at the transcriptional level [6]. Accumulating data are starting to reveal the roles of protein coding genes in the regulatory networks of FoxM1. However, the lncRNA genes regulated by FoxM1 remain obscure. Increasing evidence indicates that lncRNAs are multifaceted regulators of various cellular processes, with roles that include influencing epigenetic landscapes, transcriptional circuitry, and post-transcriptional regulatory processes [16, 38]. In the present study, we identified two lncRNAs (Snhg8 and Gm26917) that mediated the function of FoxM1 in regulating SC proliferation and survival, respectively.

Figure 6. Snhg8 sustained SC proliferation via promoting the transcription of ribosome proteins. (A): Downregulated mRNA of ribosome protein genes in FoxM1-knockdown proliferative C2C12 cells (data from RNA-Sequencing, p < .05). (B): qPCR analysis of ribosome protein gene expression (Rps10, Rps16, Rps27, Rpl10a, Rpl26, and Rpl36) in FoxM1 knockout SCs, n = 4. (C): qPCR analysis of ribosome protein gene expression in C2C12 cells (proliferative state) with the expression of Snhg8 (pCDH-Snhg8) or control vector (pCDH), n = 3. (E, F): The effect of overexpression of Snhg8 on the proliferation of FoxM1-knockout C2C12 cells was analyzed by flow cytometry using BrdU-FITC and SSC (left panel). The histogram depicts the frequency of BrdU+ cells (right panel), n = 3. (G): The effect of overexpression or knockdown of Snhg8 and Gm26917 on the proliferation of FoxM1-knockout SCs was determined by BrdU incorporation analysis, n = 4. *, p < .05; **, p < .01. The results are expressed as means ± SD. Abbreviations: BrdU, bromodeoxyuridine; FITC, fluorescein isothiocyanate; FoxM1, forkhead box M1; qPCR, quantitative polymerase chain reaction; SC, satellite cells; SSC, side scatter.
are direct targets of FoxM1 in SCs. Mechanistically, we demonstrated that Snhg8 affects ribosome protein gene transcription and that the FoxM1-Snhg8 axis is necessary for the proliferation of SCs. Recently, a novel lncRNA, SLERT, was shown to enhance pre-ribosome RNA transcription via an interaction with RNA helicase DDX21 to promote RNA polymerase I transcription [39]. In the present study, we revealed that another lncRNA (Snhg8) significantly affected the transcription of ribosome proteins (FoxM1 may also directly regulate the transcription of ribosome proteins), which are crucial for the structural integrity of the ribosome. Transcriptional regulation of ribosome proteins is synergistically stimulated by the transcriptional factors SP1 and CREB [40], and we inferred the putative interaction of Snhg8 and the above transcription factors. Ribosome biogenesis is a vital process for cell proliferation [41]; further study is required to explore whether Snhg8 ubiquitously regulates the transcription of ribosome proteins and the underlying mechanisms.

The mechanisms by which IncRNAs regulate apoptosis and cellular survival have not been clarified; however, it is well established that IncRNAs frequently function as sponges for miRNAs [15]. We identified Gm26917 as a novel IncRNA target of FoxM1 in both quiescent and proliferative C2C12 cells, with a high possibility of functioning as an miRNA sponge. Notably, we discovered that miR-29b (a predicted target of Gm26917) showed increased expression in both FoxM1-knockdown C2C12 cells and FoxM1-cKO SCs. MiR-29b has been demonstrated to mediate several muscle diseases, including muscle sarcopenia and atrophy [34, 35]; however, the impact of miR-29b on SC survival remains unclear. We demonstrated that upregulation of miR-29b resulted in increased apoptosis in both C2C12 cells and SCs. IGF1 and PI3K are direct targets of miR-29b [35]. Consistently, we also found that the expression of IGF1 was obviously inhibited in quiescent and activated FoxM1-knockdown C2C12 cells, as well as the corresponding mutant Gm26917 are indicated in the upper panel. (G, H): Overexpression of Gm26917 (pCDH-Gm26917), but not mutant Gm26917 (pCDH-mut Gm26917) reduced the apoptosis of FoxM1-knockdown C2C12 cells (proliferative state), n = 3. (I): The effect of overexpression or knockdown of Snhg8 and Gm26917 on the apoptosis of FoxM1-knockout SCs was determined by Annexin V/PI staining and analyzed by flow cytometry, n = 5. *, p < .05; **, p < .01. The results are expressed as means ± SD. Abbreviations: APC, allophycocyanin; FoxM1, forkhead box M1; miRNA, MicroRNAs; PE, phycoerythrin; qPCR, quantitative polymerase chain reaction; SC, satellite cells.

Figure 7. Gm26917 was necessary for SC survival via sponging miR-29b. (A): qPCR analysis of putative miRNAs (miR-425, miR486, miR-28, miR-29b, miR-125, and miR-320) targeting Gm26917 in C2C12 cells, n = 3. (B): qPCR analysis of miR-29b expression in FoxM1 knock-out SCs, n = 4. (C, D): Representative histograms (C) of flow cytometry analysis of apoptosis in C2C12 cells and SCs (isolated from wild-type C57BL/6J) with the expression of miR-29b (pCDH-miR-29b) or control vector (pCDH). The histogram (D) depicts the frequency of AnnexinV+/PI cells in C2C12 cells and SCs with the expression of miR-29b or control vector, n = 4. (E): qPCR analysis of miR-29b in Gm26917 knockdown (shGm26917) C2C12 cells and control cells, n = 5. (F): The effect of overexpression of the wild-type and mutant Gm26917 on the expression of miR-29b in C2C12 cells (proliferative state), n = 3. The predicted base-pairing sequence between Gm26917 and miR-29b, as well as the corresponding mutant Gm26917 are indicated in the upper panel. (G, H): Overexpression of Gm26917 (pCDH-Gm26917), but not mutant Gm26917 (pCDH-mut Gm26917) reduced the apoptosis of FoxM1-knockdown C2C12 cells (proliferative state), n = 3. (I): The effect of overexpression or knockdown of Snhg8 and Gm26917 on the apoptosis of FoxM1-knockout SCs was determined by Annexin V/PI staining and analyzed by flow cytometry, n = 5. *, p < .05; **, p < .01. The results are expressed as means ± SD. Abbreviations: APC, allophycocyanin; FoxM1, forkhead box M1; miRNA, MicroRNAs; PE, phycoerythrin; qPCR, quantitative polymerase chain reaction; SC, satellite cells.
Conclusions

Our findings indicated an important role for FoxM1 in the maintenance of SCs, and deregulation of FoxM1 expression leads to impaired proliferation and survival of SCs. We further suggested that the FoxM1-Snhg8 and FoxM-Gm26917 axes sustain the proliferation and survival of SCs, respectively, in a tissue-specific manner. Our study revealed IncRNA genes that are regulated by FoxM1, and provides insights into the molecular network of SC regulation.

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Author Contributions

Z.C., N.B., X.Q., Z.Z., Y.S.; and Z.L.: performed the experiments and/or data analyses. Z.Q., J.C.: and Y.H.: designed the research. Z.C.: and Y.H.: prepared the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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