LncRNA SMARCD3-OT1 Promotes Muscle Hypertrophy and Fast-Twitch Fiber Transformation via Enhancing SMARCD3X4 Expression

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Abstract: Long noncoding RNA (lncRNA) plays a crucial part in all kinds of life activities, especially in myogenesis. SMARCD3 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3) is a member of the SWI/SNF protein complex and was reported to be required for cell proliferation and myoblast differentiation. In this study, we identified a new lncRNA named SMARCD3-OT1 (SMARCD3 overlapping lncRNA), which strongly regulated the development of myogenesis by improving the expression of SMARCD3X4. We overexpressed and knockdown the expression of SMARCD3-OT1 and SMARCD3X4 to investigate their function on myoblast proliferation and differentiation. Cell experiments proved that SMARCD3-OT1 and SMARCD3X4 promoted myoblast proliferation through the CDKN1A pathway and improved differentiation of differentiated myoblasts through the MYOD pathway. Moreover, they upregulated the fast-twitch fiber-related genes and downregulated the slow-twitch fiber-related genes, which indicated that they facilitated the slow-twitch fiber to transform into the fast-twitch fiber. The animals’ experiments supported the results above, demonstrating that SMARCD3-OT1 could induce muscle hypertrophy and fast-twitch fiber transformation. In conclusion, SMARCD3-OT1 can facilitate slow-twitch fibers to transform into fast-twitch fibers.

Keywords: lncRNA; myogenesis; myoblast; cell proliferation; cell differentiation; CDKN1A; MYOD; SMARCD3

1. Introduction

Myogenesis is a highly ordered process. A series of myogenic factors regulates basic activities inside the muscle. The number of skeletal muscles is almost fixed in utero, and the key to muscle weight gain is the size of myofibers, which can be changed with exercise [1,2]. In addition, skeletal muscle is dynamic muscle tissue. Under certain conditions, fast-twitch fibers and slow-twitch fibers can be transformed into each other. Different types of muscle fibers have different functional, biochemical, and morphological characteristics [3].

Although lncRNAs have similar parts with mRNA, lncRNAs have more flexible regulation methods and more significant differences in expression between tissues [4–6]. These characteristics make the study of lncRNA more difficult and complex. Recently, more and more lncRNAs were found to be relevant to epigenetic and transcriptional regulation in
myogenesis [7–10]. Studies have shown that some lncRNAs could activate the expression of adjacent encoding genes to perform different functions on life activities [11,12].

The protein encoded by the SMARCD3 gene is a member of the SWI/SNF protein family. Its members have helicase and ATPase activities and can regulate the transcription of genes by changing the chromatin structure around the genes, playing an important role in physiological processes [13–15]. Reports show that SMARCD3 can regulate the cell cycle through the CDKN1A signaling pathway, affecting tumor formation [16], and it can also regulate muscle development in zebrafish through MYOD and MYF5 signaling pathways [17,18]. However, the specific molecular mechanism of SMARCD3 is still unknown.

RNA-sequencing based on the pectoral muscle and soleus of Xinghua chicken was performed (ID 751251-BioProject-NCBI (nih.gov)/accession number PRJNA751251/accessed date: 1 September 2021) in the previous experiment. Based on these results, a new lncRNA, MSTRG.2872.11, partly overlays on a functional gene named SMARCD3X4 and is identified and named SMARCD3-OT1. SMARCD3-OT1 is found highly expressed in skeletal muscle and upregulated in myoblast differentiation. In vitro, overexpression and knockdown experiments show that SMARCD3-OT1 promotes myoblast proliferation during the proliferation stage and improves myoblast differentiation during the differentiation stage. In vivo, SMARCD3-OT1 can facilitate slow-twitch fiber transfer to fast-twitch fiber and induce muscle hypertrophy. The research reveals that SMARCD3-OT1 promotes the expression of SMARCD3X4 in both mRNA and protein levels, improving myoblast proliferation and myotube differentiation through the CDKN1A pathway and MYOD pathway, respectively. Overall, our studies identify a novel lncRNA, which regulates myogenesis in myoblasts through post-transcriptional gene regulation and affects the transformation of myofibers, providing us a new therapeutic target of muscle atrophy therapy.

2. Results

2.1. Identification and Characterization of SMARCD3-OT1

For exploring the function of SMARCD3-OT1, the 5′ and 3′ ends of SMARCD3-OT1 were amplified by the rapid amplification of cDNA ends (RACE) assay (Figure 1a). The sequence of SMARCD3-OT1 is offered in Table S1. The National Center for Biotechnology Information was used to confirm the position of SMARCD3-OT1. The results showed that SMARCD3-OT1 consisted of 1542 bases and was located at chicken chromosome 2 (chr2: 118054–118633, 133484–133684, 134874–135641). The results also revealed that SMARCD3-OT1 partly overlaps on the 5′ untranslated region (UTR) of the mRNA SMARCD3X4 (Figure 1b). A prediction was performed in Coding Potential Calculator [19] (Figure 1c) to predict the coding ability of lncRNA SMARCD3-OT1. Moreover, the termination codon of the ORFs of SMARCD3-OT1 and the initiation codon of the EGFP gene were mutated to construct a series of ORFs-EGFP fusion protein vectors, which are subsequently transfected into chicken’s primary myoblasts (CPMs). The sequences of ORFs are listed in Table S2. The proteins acquired from those cells were used for Western blot, which proved that SMARCD3-OT1 did not have coding ability (Figure 1d,e).

In the previous RNA sequencing (ID 751251-BioProject-NCBI (nih.gov)/accession number PRJNA751251/accessed date: 1 September 2021), SMARCD3-OT1 was found highly expressed in pectoral muscle, and the subsequent quantitative real-time polymerase chain reaction (qRT-PCR) results proved this tendency (Figure 1f,g). The expression of SMARCD3-OT1 was found to keep a steady level in the proliferation stage (CPMs cultured in growth medium (GM)) and was significantly upregulated during myoblast differentiation (CPMs cultured in differentiation medium (DM)) (Figure 1h). Otherwise, SMARCD3-OT1 was highly expressed in breast muscle and leg muscle, indicating that they may participate in the myogenesis of skeletal muscle (Figure 1i). Meanwhile, the expression levels of SMARCD3-OT1 increased from embryonic day 10 (E10) to E15 (Figure 1j). Cell-fractionation assays suggested that SMARCD3-OT1 mainly existed in the nuclei of CPMs (Figure 1k,l).
Figure 1. Identification of lncRNA SMARCD3-OT1 and the expression pattern of SMARCD3-OT1. (a) Results of SMARCD3-OT1 5’ RACE and 3’RACE. (b) Schematic image of the locations for SMARCD3-OT1 (red and blue) and SMARCD3X4 (red and black). Arrows represent the direction of transcription. (c) Prediction of the SMARCD3-OT1 protein-coding ability. (d) Western blotting with anti-EGFP. (e) Diagram of the EGFP fusion construct vectors used for transfection. The initiation codon ATGGTG of the EGFP (EGFP wt) gene is mutated to ATTGTT (EGFP mut), and the termination codon TGA of ORFs is mutated to TGG. (f,g) RNA sequencing result of SMARCD3-OT1 and further qRT-PCR verification. (h) Expression levels of SMARCD3-OT1 in CPMs cultured in growth medium at 50% and 100% cell confluency (50%GM and 100%GM) and differentiation medium from 1 to 5 days (DM1 to DM5) (n = 6). (i) Tissue expression profiles of SMARCD3-OT1 in 7-week-old chickens (n = 6). (j) Expression levels of SMARCD3-OT1 from E10 to E15 (n = 6). (k,l) Distribution of SMARCD3-OT1 in the cytoplasm and nuclei of CPMs was determined by PCR and qRT-PCR. Data are presented as mean ± SEM. Statistical significance of differences between means was assessed using an independent sample t test (*p < 0.05; **p < 0.01; N.S., no significant difference).

2.2. SMARCD3-OT1 Promotes the Proliferation of Myoblasts

CPMs were transfected with the overexpression vector or the antisense oligonucleotides (ASO) fragment of SMARCD3-OT1 when the CPMs achieved 80–90% confluence.
After 48 h, the cells were collected and used for subsequent experiments. The fold-changes of SMARCD3-OT1 were measured by qRT-PCR (Figure S1a,b). The results obtained from the qRT-PCR are presented in that overexpression of SMARCD3-OT1 significantly downregulated the expression level of CDKN1A, an upstream inhibitory gene of the cell cycle. Then, SMARCD3-OT1 upregulated the cell-cycle related genes (CCNK1B, CCNA1, CCNE, and CCND1), improving myoblast proliferation. On the contrary, knockdown of SMARCD3-OT1 increased CDKN1A expression and inhibited other cell-cycle-related genes, repressing the proliferation of myoblasts. (Figure 2a,b). The cell cycle phase assay suggested that SMARCD3-OT1 prolonged the S phase of cells, preventing cells from entering the G2M phase and promoting the proliferation of cells (Figure 2c,d). In order to further assess the function of SMARCD3-OT1, the cell counting kit-8 (CCK-8) assay was performed to measure the proliferation condition of myoblasts after transfection. It can be seen from these data that overexpression of SMARCD3-OT1 significantly improved cell proliferation after 36 h of transfection, while inhibition of SMARCD3-OT1 repressed cell proliferation at 48 h (Figure 2e,f). These results indicated that SMARCD3-OT1 regulated the cell cycle in the early stage of cell proliferation. In addition, the 5-ethynyl-2′-deoxyuridine (EdU) assay was used to verify the results above. Distinct improvement of cell proliferation was observed after overexpression of SMARCD3-OT1, and the proportion of proliferating cells was increased under the regulation of SMARCD3-OT1. By contrast, the knockdown of SMARCD3-OT1 had inverse results (Figure 2g–j). These data suggest that SMARCD3-OT1 improves myoblast proliferation during myoblast proliferation stage (48 h after CPMs achieved 80–90% confluence).

2.3. SMARCD3-OT1 Promotes the Differentiation of Myoblasts during the Differentiation Stage, Facilitating the Myofiber Transformation

After 48 h transfection, CPMs were induced to differentiation for 3 days using the differentiated medium. After 3 days of differentiation, the myoblasts fused together, and myotubes were formed. Then, the expression levels of differentiation marker genes, including MyHC, MYOD, MYOG, and MYF5, were detected by qRT-PCR and Western blot to assess the function of SMARCD3-OT1 on differentiation. Overexpression of SMARCD3-OT1 significantly increased the expression level of these differentiation-related genes in mRNA level, while knockdown of SMARCD3-OT1 had inverse results (Figure 3a,b). At the protein level, SMARCD3-OT1 improved these genes’ expression level as well (Figure 3c,d). Moreover, immunofluorescence staining was performed to investigate the function of SMARCD3-OT1 in differentiation. The results demonstrated that overexpression of SMARCD3-OT1 could promote the differentiation of myoblasts and the formation of myotubes, and knockdown of SMARCD3-OT1 had a negative influence on myoblast differentiation and myofiber mature (Figure 3e–h). These data demonstrate that SMARCD3-OT1 improves myoblast differentiation after 3 days of differentiation induction, which means that SMARCD3-OT1 has different functions in the myoblast proliferation and differentiation stage.
Figure 2. SMARCD3-OT1 promotes the proliferation of myoblasts. (a,b) Relative mRNA levels of several cell cycle genes after overexpression or knockdown of SMARCD3-OT1 (n = 6). (c,d) Cell cycle analysis of CPMs at 48 h after overexpression or knockdown of SMARCD3-OT1 (n = 4). (e,f) CCK-8 assays were performed in CPMs with SMARCD3-OT1 overexpression or knockdown (n = 6). (g,h) After 48 h transfection of overexpression vector or ASO of SMARCD3-OT1, CPMs were stained by EdU and Hoechst, and the images were captured by a fluorescence microscope (n = 6). (i,j) Proliferation rate of CPMs with SMARCD3-OT1 overexpression or knockdown (n = 6). All proliferation-related experiments are performed in CPMs during the cells’ proliferation stage without myoblast fusion. Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using an independent sample t test. *p < 0.05, **p < 0.01.
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Figure 3. SMARCD3-OT1 promotes the differentiation of myoblasts during the differentiation stage, facilitating myofiber transformation. (a,b) Relative mRNA levels of several cell differentiation marker genes after overexpression or knockdown of SMARCD3-OT1 (n = 6). (c,d) Relative protein levels of several cell differentiation marker genes after overexpression or knockdown of SMARCD3-OT1 (n = 3). The numbers shown below the bands were folds of band intensities relative to control. Band intensities were quantified by ImageJ and were normalized to GAPDH. (e,f) MyHC immunostaining of CPMs transduced with SMARCD3-OT1 overexpression or knockdown. Cells were differentiated for 72 h after transfection. The nuclei were visualized with 4′,6-diamidino-2-phenylindole buffer (DAPI) (n = 6). (g,h) Myotube area (%) of CPMs transduced with SMARCD3-OT1 overexpression or knockdown in immunofluorescence assay (n = 6). (i,j) Relative mRNA levels of several fast-twitch fiber- and slow-twitch fiber-related genes after overexpression or knockdown of SMARCD3-OT1 (n = 6). All differentiation-related experiments are performed in CPMs after 3 days of differentiation induction using the differentiated medium. Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using an independent sample t test. * p < 0.05, ** p < 0.01.

Otherwise, we also found that SMARCD3-OT1 improved the expression of fast-twitch fiber-related genes (TNNT3, TNNC2, and SRL) and inhibited the slow-twitch fiber-related genes (TNNT1, TNNC1, and SRL).
genes (TNNI1, TNNT1, and TNNC1) (Figure 3i,j). These results indicate that SMARCD3-OT1 facilitates slow-twitch fibers to transfer to fast-twitch fibers.

2.4. SMARCD3-OT1 Positively Regulated SMARCD3X4 Transcriptional Activity

Because SMARCD3-OT1 is located in the 5′ UTR of SMARCD3X4, we hypothesized that SMARCD3-OT1 might regulate SMARCD3X4 by the cisregulating way (Figure 1b). First, we investigated the expression pattern of SMARCD3X4. The data revealed that SMARCD3X4 was highly expressed in early cell differentiation, and SMARCD3X4 was highly expressed in breast muscle and leg muscle (Figure S2a,b). Otherwise, the expression of SMARCD3X4 increased from E13 and sharply increased in E15 (Figure S2c). RNA of the nucleus and cytoplasm were separated and used to measure the expression of SMARCD3X4 in the cell. The data revealed that SMARCD3X4 mainly existed in the nucleus (Figure S2d,e). The results above illustrate a similar expression pattern between SMARCD3X-OT1 and SMARCD3X4, which indicates the relationship between SMARCD3X-OT1 and SMARCD3X4.

The qRT-PCR results showed that the mRNA expression level of SMARCD3X4 was upregulated with the overexpression of SMARCD3-OT1 and downregulated with the knockdown of SMARCD3-OT1 in CPMs (Figure 4a,b). The results of the Western blot also proved that the protein level of SMARCD3X4 was positively correlated with SMARCD3-OT1 in CPMs (Figure 4c,d). In addition, the experiments in vivo proved this tendency as well (Figure 4e–h). To further ensure the functional fragment of SMARCD3-OT1, the full-length sequence of SMARCD3-OT1 was divided into five fragments as follows: E1 (1–768 bp), E2 (769–964 bp), E3 (965–1542 bp), E1 + 2 (1–964 bp), and E1 + 2 + 3 (1–1542 bp). These fragments were inserted into the 5′ UTR of the luciferase gene and then transfected in the DF-1 cell line (Figure 4i). The luciferase assay showed that fragment E3 significantly improved luciferase activity, while fragment E1 + 2 significantly inhibited luciferase activity. The data indicated that fragment E3 might play a positive role in the expression of SMARCD3X4, while fragment E1 + 2 has a negative influence on the expression of SMARCD3X4. Fragment E1 + 2 + 3 finally improved the luciferase activity, hinting that the function of fragment E1 + 2 was neutralized by fragment E3 (Figure 4j). Moreover, after co-transfected exon fragments with overexpression vector or ASO of SMARCD3-OT1 in DF-1 cell line, the dual-luciferase assays proved that SMARCD3-OT1 could improve the effects of exon 3 and exon 1 + 2 + 3, and they inhibited the negative effect of exon 1 + 2 (Figure 4k,l). Overall, the full-length of SMARCD3-OT1 ultimately showed a positive regulation toward SMARCD3X4, suggesting that SMARCD3-OT1 may recruit transcriptional factors to the promoter of SMARCD3X4, thus promoting the transcriptional activity of SMARCD3X4.
Figure 4. SMARCD3-OT1 positively regulated SMARCD3X4. (a,b) mRNA expression levels of SMARCD3X4 in CPMs with SMARCD3-OT1 overexpression or knockdown (n = 6). (c,d) Protein expression levels of SMARCD3X4 in CPMs with SMARCD3-OT1 overexpression or knockdown (n = 3). The numbers shown below the bands are folds of band intensities relative to control. Band intensities were quantified by ImageJ and normalized to GAPDH. (e,f) mRNA expression levels of SMARCD3X4 in animals with SMARCD3-OT1 overexpression or knockdown (n = 6). (g,h) Protein expression levels of SMARCD3X4 in animals with SMARCD3-OT1 overexpression or knockdown (n = 3). The numbers shown below the bands are folds of band intensities relative to control. Band intensities were quantified by ImageJ and normalized to GAPDH. (i) Sketch of the PGL3-promoter vector inserted with SMARCD3-OT1 fragments. (j) After 48 h, the relative luciferase activities of DF-1 cell transfected with a different recombinant vector were detected (n = 8). (k,l) Relative luciferase activities of DF-1 cell co-transfected with different recombinant vectors and overexpression vector or ASO of SMARCD3-OT1. Data are presented as mean ± SEM. Statistical significance of differences between means was assessed using an independent sample t test. *p < 0.05, **p < 0.01.
To investigate the potential transcriptional factor, we used the hTFTarget database [20] to screen for the transcriptional factors that could combine with the 2,000 bp upstream of SMARCD3X4 (Table S3). Then, the RIPseq website [21] was used to evaluate the potentialities of the combination between SMARCD3-OT1 and these transcriptional factors. The results showed that the transcriptional factor SP2 had the potential to combine with the SMARCD3-OT1 and 2,000 bp upstream of SMARCD3X4 (Figure S3a,b). These results indicate that SP2 may be the regulator that participates in the mechanism between SMARCD3-OT1 and SMARCD3X4.

2.5. SMARCD3X4 Positively Regulates Myoblasts Proliferation

After 48 h transfection, the fold-changes in mRNA and protein levels were measured by qRT-PCR and Western blot assays (Figure S4a–d). Being similar to SMARCD3-OT1, overexpression of SMARCD3X4 significantly inhibited the expression level of CDKN1A and improved the expression of CDKN1B, CCNA1, CCNE1, and CCND1. In addition, the knockdown of SMARCD3X4 had an inverse influence, revealing its positive influence on cell proliferation (Figure 5a,b). The cell cycle phase assay was performed to investigate the function mentioned above. After overexpression of SMARCD3X4, the number of cells in the S phase increased remarkably. Meanwhile, after the knockdown of SMARCD3X4, the number of cells in the S phase decreased, and the number of cells in the G2M phase showed an increase. These data demonstrated that SMARCD3X4 improved cell proliferation by stopping cells from entering the G2M phase (Figure 5c,d). In addition, we performed the EdU assay to verify this conclusion. After 48 h transfection, the number of proliferating cells significantly increased with the overexpression of SMARCD3X4 and decreased with the inhibition of SMARCD3X4 (Figure 5e–h). Otherwise, the CCK-8 assay showed that SMARCD3X4 promoted cell proliferation after 48 h transfection (Figure 5i,j). These data reveal that SMARCD3X4 positively regulates myoblast proliferation after 48 h transfection.

2.6. SMARCD3X4 Positively Regulates Myoblasts Differentiation and Accelerates Myofiber Transformation

With respect to cell differentiation, qRT-PCR was used to detect the expression of differentiation-related genes with overexpression or inhibition of SMARCD3X4 in CPMs after 3 days of differentiation. From the data, we observed that overexpression of SMARCD3X4 could improve the expression of MYOD, MYOG, MyHC, and MYF5, and inhibition of SMARCD3X4 repressed the expression level of these genes (Figure 6a,b). Similarly, at the protein level, SMARCD3X4 expression significantly improved the expression of differentiation marker genes, promoting the differentiation of myoblasts through the MYOD pathway in differentiated myoblasts (Figure 6c,d). Meanwhile, the immunofluorescence staining assay illustrated that the cells overexpressed with SMARCD3X4 had bigger myofibers, while the cell inhibited with SMARCD3X4 showed smaller myofibers. These results demonstrated that SMARCD3X4 facilitated the formation and maturity of the myofiber (Figure 6e–h). These cell experiments reveal that SMARCD3X4 promotes myoblast differentiation after 3 days of differentiation, indicating that SMARCD3X4 plays different roles in myoblast proliferation and differentiation stages. This result is similar to that of SMARCD3-OT1.

Otherwise, the qRT-PCR results also suggested that SMARCD3X4 facilitated myofibers to transfer from slow-twitch myofibers to fast-twitch myofibers. Overexpression of SMARCD3X4 increased the expression of fast-twitch myofiber-related genes and decreased the expression of slow-twitch myofiber-related genes. By contrast, the knockdown of SMARCD3X4 showed a reverse influence (Figure 6i,j).
Figure 5. SMARCD3X4 promotes cell proliferation. (a,b) Relative mRNA levels of several cell cycle genes after overexpression or knockdown of SMARCD3X4 (n = 6). (c,d) Cell cycle analysis of CPMs after overexpression or knockdown of SMARCD3X4 (n = 4). (e,f) EdU proliferation assays for CPMs with the overexpression or knockdown of SMARCD3X4 (n = 6). (g,h) Proliferation rate of CPMs with SMARCD3X4 overexpression or knockdown (n = 6). (i,j) CCK-8 assays were performed in CPMs with SMARCD3X4 overexpression or knockdown (n = 6). All proliferation-related experiments are performed in CPMs during the cells’ proliferation stage without myoblast fusion. Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using an independent sample t test. * p < 0.05, ** p < 0.01.
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These cell experiments reveal that SMARCD3X4 promotes myoblast differentiation after 3 days of differentiation, indicating that SMARCD3X4 plays different roles in myoblast proliferation and differentiation stages. This result is similar to that of SMARCD3-OT1.

Figure 6. SMARCD3X4 promotes cell differentiation through the MYOD pathway and accelerates myofiber transformation during myoblast differentiation. (a, b) Relative mRNA levels of several cell differentiation marker genes after overexpression or knockdown of SMARCD3X4 (n = 6). (c, d) Relative protein levels of several cell differentiation marker genes after overexpression or knockdown of SMARCD3X4 (n = 3). The numbers shown below the bands are folds of band intensities relative to control. Band intensities were quantified by ImageJ and normalized to GAPDH. (e, f) MyHC immunostaining of CPMs with SMARCD3X4 overexpression or knockdown. (g, h) Myotube area (%) of CPMs transduced with SMARCD3X4 overexpression or knockdown in immunofluorescence assay (n = 6). (i, j) Relative mRNA levels of several fast-twitch fiber- and slow-twitch fiber-related genes after overexpression or knockdown of SMARCD3X4 (n = 6). All differentiation-related experiments are performed in CPMs after 3 days of differentiation induction using the differentiated medium. Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using an independent sample t test. *p < 0.05, **p < 0.01.
2.7. SMARCD3-OT1 Regulates CDKN1A Pathway and MYOD Pathway In Vivo

The gastrocnemius muscles injected with overexpression lentivirus or modified ASO fragments of SMARCD3-OT1 were detached and used for further experiments. The samples were detected by qRT-PCR; then the samples that were successfully overexpressed, or knockdown of SMARCD3-OT1, were collected and prepared for subsequent experiments.

Seven-day-old chickens were induced (LV-SMARCD3-OT1) or knockdown (LV-NC) SMARCD3-OT1 by lentivirus and specially modified ASO in order to investigate the role of SMARCD3-OT1. qRT-PCR was performed to detect the effect of lentivirus injection. As can be seen from Figure 7a,b, the fold changes of LV-SMARCD3-OT1 and ASO are 28.7 and 0.3, respectively. qRT-PCR and Western blot were used to detect the expression of genes related to the CDKN1A pathway and MYOD pathway in vivo. The data showed that overexpression of SMARCD3-OT1 downregulated the expression of CDKN1A and upregulated the expression of the downstream genes of CDKN1A in vivo, and the knockdown of SMARCD3-OT1 had different results. Together, these data indicated that SMARCD3-OT1 still regulated the CDKN1A pathway in vivo (Figure 7c,d). In addition, the qRT-PCR and Western blot results suggested that the myogenesis maker genes expressions increased with the expression of SMARCD3-OT1 in mRNA and protein levels, indicating that SMARCD3-OT1 still regulated the MYOD pathway in vivo (Figure 7e–h).

![Figure 7. SMARCD3-OT1 regulates the CDKN1A pathway and MYOD pathway in vivo. (a) Relative SMARCD3-OT1 expression in gastrocnemius muscle after infection with SMARCD3-OT1-expressing lentivirus (LV-SMARCD3-OT1) or negative control (LV-NC) (n = 6). (b) Relative SMARCD3-OT1 expression in gastrocnemius muscle after infection with modified ASO specific to SMARCD3-OT1 (ASO-SMARCD3-OT1) or negative control (NC) (n = 6). (c,d) Relative mRNA levels of several cell cycle genes after overexpression or knockdown of SMARCD3-OT1 in vivo (n = 6). (e,f) Relative mRNA levels of several myogenesis marker genes after overexpression or knockdown of SMARCD3-OT1 in vivo (n = 6). (g,h) Relative protein levels of several myogenesis marker genes after overexpression or knockdown of SMARCD3-OT1 in vivo (n = 3). The numbers shown below the bands are folds of band intensities relative to control. Band intensities were quantified by ImageJ and normalized to GAPDH. Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using a paired sample t test. * p < 0.05, ** p < 0.01.](image-url)
2.8. SMARCD3-OT1 Positively Regulates Muscle Development and Improves Muscle Hypertrophy and Myofibers Transformation

To further investigate the function of SMARCD3-OT1 in myogenesis, the mass of the muscle injected with overexpressed lentivirus or modified ASO was measured. The data showed that the overexpression of SMARCD3-OT1 led to an increased mass of leg muscle (Figure 8a). On the contrary, the knockdown of SMARCD3-OT1 resulted in a decrease in leg muscle (Figure 8b). Meanwhile, the muscle was sliced up and used for hematoxylin and eosin (H&E) staining. The results revealed that muscle induced with SMARCD3-OT1 had bigger myofibers, and the proportion of myofibers with a larger diameter was increased. By contrast, the muscle injected with modified ASO had smaller myofibers, and the proportion of myofiber with a smaller diameter was increased (Figure 8c–e). These data demonstrated that SMARCD3-OT1 induced muscle hypertrophy and improved muscle development.

Immunohistochemical assays proved that SMARCD3-OT1 enhanced the expression level of MYH1, a fast-twitch myofiber protein. The proportion of myofibers stained with MYH1 increased with the overexpression of SMARCD3-OT1 and decreased with the inhibition of SMARCD3-OT1 (Figure 8f,h,i). Conversely, the overexpression of SMARCD3-OT1 decreased the proportion of myofibers stained with MYH7, a slow-twitch myofiber protein, and the inhibition of SMARCD3-OT1 increased this proportion (Figure 8g,j,k). These data demonstrated the inhibitory effect of SMARCD3-OT1 on MYH7. LDH is one of the important enzymes in anaerobic glycolysis and gluconeogenesis, which can reflect the level of anaerobic glycolytic capacity of skeletal muscle. The activity of LDH reflects the process of fast-twitch fiber transformation. The data showed that the activity of lactate dehydrogenase (LDH) was increased with the SMARCD3-OT1 induced and suppressed with the knockdown of SMARCD3-OT1. These results indicated that SMARCD3-OT1 facilitated the capacity of anaerobic glycolysis in skeletal muscle and promoted fast-twitch myofiber transformation (Figure 8l,m). Otherwise, Western blot was used to qualify the MYH1 and MYH7 protein content, which also revealed the positive influence of SMARCD3-OT1 on the process of fast myofiber transformation (Figure 8n,o). The qRT-PCR results of myofiber transformation-related genes also proved this conclusion (Figure 8p,q).
Figure 8. SMARCD3-OT1 positively regulates muscle development and improves muscle hypertrophy and myofiber transformation. (a,b) Relative mass of gastrocnemius muscles after injection with overexpression or knockdown of SMARCD3-OT1 in vivo (n = 15). (c–e) H&E staining and frequency distribution of fiber cross-section area. (f–h) MYH1 and MYH7 expression levels. (i,j) The percentage of myofibers with MYH1 in each group. (k,l) The percentage of myofibers with MYH7 in each group. (m) Relative LDH activity. (n) Western blot analysis of MYH1, MYH7, and GAPDH. (o) qRT-PCR analysis of MYH1, MYH7, and GAPDH. (p,q) Relative mRNA expression of TNNT3, TNNT2, SRL, TNNH1, TNNT1, and TNHC1.
with overexpression or knockdown of SMARCD3-OT1 in vivo (n = 15). (c–e) H&E staining and frequency distribution of fiber cross-section areas in gastrocnemius muscle with SMARCD3-OT1 overexpression or knockdown (n = 6). (f,h,i) Immunohistochemistry of MYH1 and the percentage of myofibers stained with MYH1 protein with SMARCD3-OT1 overexpression or knockdown (n = 6). (g,j,k) Immunohistochemistry of MYH7 and the percentage of myofibers stained with MYH7 protein with SMARCD3-OT1 overexpression or knockdown (n = 6). (l,m) Relative enzymes activity of LDH in gastrocnemius muscle with SMARCD3-OT1 overexpression or knockdown (n = 6). (n,o) Relative protein level of MYH1 and MYH7 in SMARCD3-OT1-inducd or knockdown muscle (n = 3). The numbers shown below the bands are folds of band intensities relative to control. Band intensities were quantified by ImageJ and normalized to GAPDH. (p,q) Relative mRNA levels of several fast-twitch fiber- and slow-twitch fiber-related genes after overexpression or knockdown of SMARCD3-OT1 in vivo (n = 6). Data are expressed as a fold change relative to the control. Results are shown as mean ± SEM. Statistical significances of differences between means were assessed using a paired sample t test. * p < 0.05, ** p < 0.01.

3. Discussion

LncRNA plays an important role in various biological growth processes, affecting life processes, including bone development, muscle growth, fat deposition, disease resistance, tumorigenesis [22–29], and so on. Research about the regulatory mechanism of LncRNAs mainly focused on the competing endogenous RNAs way, in which LncRNA competitively bound the target gene of miRNA to reduce the effect of miRNA on target mRNA and positively regulated the expression of target genes [30–33]. However, reports about the relationship between LncRNAs and their adjacent functional genes are relatively rare.

SMARCD3X4 is one of the transcripts of the SMARCD3 gene, and the protein coded by SMARCD3 is one of the components of the SWI/SNF complex, which is an evolutionarily conserved multi-subunit chromatin-remodeling complex, using the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin [34]. The SWI/SNF complex can regulate transcription of target genes, regulating cell proliferation and differentiation [35–37].

In this study, we proved that SMARCD3-OT1 can positively regulate the expression of SMARCD3X4, and the fragment E3 (965–1542) of SMARCD3-OT1 is the main positive regulator of SMARCD3X4. The overexpression of SMARCD3-OT1 significantly improves SMARCD3X4 expression on the mRNA and protein level, which indicates that SMARCD3-OT1 may influence mRNA transcription or translation of SMARCD3X4 to some extent. We found that the transcriptional factor SP2 is the potential to combine with both LncRNA SMARCD3-OT1 and 2000 bp upstream of SMARCD3X4 by using the target database and RIPseq website; thus, we speculated that SP2 may be the regulator that participates in the mechanism between SMARCD3X4 and LncRNA SMARCD3-OT1.

Cell proliferation and differentiation are two different stages of cell processes. However, some studies report that they can be improved by the same genes. Circ-RILPL1 can promote myoblast proliferation and differentiation via binding miR-145 and activating IGF1R/P13K/AKT pathway [38]. Moreover, circ-DAB1 is found to promote cell proliferation and osteogenic differentiation of human bone marrow stem cells via the RBP1/DAB1 axis [39]. In the cell experiments, we proved that SMARCD3-OT1 and SMARCD3X4 can promote myoblast proliferation in the proliferation stage (48 h after cells achieve 80–90%) and promote myoblast differentiation in the differentiation stage (3 days after differentiation induction). SMARCD3-OT1 has different functions on myoblast proliferation and differentiation. A hypothesis is that SMARCD3-OT1 regulates myoblast proliferation and differentiation depending on its intracellular concentration. SMARCD3-OT1 expression in myoblast proliferation and differentiation results in a similar expression of SMARCD3X4 in these two stages. As a component of the SWI/SNF complex, SMARCD3X4 has the potential to regulate the SWI/SNF complex. The SWI/SNF complex can change the construction of chromatin, thus regulating the activation and transcription of functional genes and controlling the process of cell proliferation and differentiation. Therefore, we speculate...
that SMARCD3-OT1 may influence the SWI/SNF complex through SMARCD3X4, playing different roles in cell proliferation and differentiation.

Although several lncRNAs have been verified as regulatory factors in cell proliferation and differentiation, few of their functions about muscle hypertrophy and myofiber remodeling have been elaborated [40]. In this study, we proved that SMARCD3-OT1 and SMARCD3X4 can promote the expression of fast-twitch fiber-related genes and inhibit the expression of slow-twitch fiber-related genes in vitro. Moreover, we demonstrated that they could improve muscle hypertrophy and fast-twitch fiber formation in vivo.

In summary, our research reveals a novel lncRNA that can positively regulate the functional gene SMARCD3X4, facilitating myoblast proliferation and differentiated myoblast differentiation, improving myotube formation and promoting muscle hypertrophy and myofiber transformation (Figure 9). These findings, which offer us new insight into lncRNA and gene SMARCD3, contribute to the development of myogenesis research.

**Figure 9.** Model of lncRNA SMARCD3-OT1 regulatory network for muscle development. Briefly, lncRNA SMARCD3-OT1 upregulates the expression of SMARCD3X4, thus promoting the proliferation of myoblasts and the differentiation of the myoblasts after 3 days of differentiation induction. In addition, lncRNA SMARCD3-OT1 improves muscle hypertrophy and fast-twitch fiber transformation in animals.

4. Materials and Methods
4.1. Ethics Statement

The animals used in the research were all fed and slaughtered under the guidance of the Institutional Animal Care and Use Committee at South China Agricultural University.
Int. J. Mol. Sci. 2022, 23, 4510 (approval ID: SCAU#2020C010, approval date: 1 June 2020). All experiments in the study were conducted under the supervision of South China Agricultural University, following international animal welfare standards.

4.2. Cell Culture and Transfection

DF-1 cell line was cultured in a growth medium consisting of Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 0.2% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) [41]. Chicken primary myoblasts (CPMs) were isolated from leg muscle detached from E11 chickens. The myoblasts isolated from leg muscle were first cultured in a 37 °C incubator for 40 min. During this time, other cells would adhere to the petri dish, and the supernatant would be transferred to another petri dish. After three operations, the pure myoblasts could be obtained [42]. The myoblasts were cultured with Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco, Carlsbad, CA, USA) supplemented with 20% FBS and 0.2% penicillin and streptomycin [43]. For inducing the differentiation of cells, the cell culture medium was replaced with a differentiated medium, RPMI 1640 medium with 2% horse serum and 0.2% penicillin and streptomycin, after which cells achieved 80–90% cell confluence. All cells were incubated by a temperature incubator with 37 °C and 5% CO₂.

All transfection operations were performed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) and were operated under the guideline of the manufacturer’s protocol.

4.3. RNA Extraction, Complementary DNA (cDNA) Synthesis, and qRT-PCR

RNA of cells and tissues was extracted by TRIZol reagent (TaKaRa, Kusatsu, Japan) following the manufacturer’s protocol. Paris Kit (Ambion, Life Technologies, Austin, TX, USA) was used in nuclear and cytoplasmic RNA fractionation experiments with the guide of the manufacturer’s protocol. cDNA was synthesized with the PrimeScript RT Reagent Kit with Genomic DNA (gDNA) Eraser (perfect real-time) (TaKaRa, Kusatsu, Japan). The cDNA used for RACE was synthesized with the SMARTer RACE cDNA Amplification Kit (Clontech, Kusatsu, Japan). The qRT-PCR experiments were performed by using the iTaq Universal SYBR Green Supermix Kit (Bio-Rad, Hercules, CA, USA) and were analyzed by Bio-Rad Real-Time Detection Machine (Bio-Rad, Hercules, CA, USA). The chicken GAPDH gene was used as an internal control. Comparative $2^{-\Delta\Delta CT}$ method was used to analyze the data from qRT-PCR [44]. Primers used in qRT-PCR are listed in Table S4.

CPMs were transfected with the overexpression vector and ASO fragment of SMARCD3-OT1 or SMARCD3X4 when the cells achieved 80–90% confluence. After 48 h, the CPMs were collected and used for the detection of cell-cycle related genes, including CDKN1A, CDKN1B, CCNA1, CCND1, and CCNE1.

After 48 h transfection, CPMs were induced to differentiation by differentiated medium for 3 days. The RNA and protein of these cells were extracted and used for the detection of differentiation marker genes, including MYOD, MYOG, MYHC, and MYF5.

4.4. 5′ and 3′ RACE

The full length of SMARCD3-OT1 was synthesized by using the SMARTer RACE cDNA Amplification Kit following the manufacturer’s protocol. The primer pairs used in RACE are listed in Table S5.

4.5. Plasmid Construction and RNA Oligonucleotides

For mutation of the pEGFP-N1 vector (Promega, Madison, WI, USA), the full length of the pEGFP-N1 vector was amplified with a mutation in the start codon (ATGGTG to ATTGTT) [45]. The mutational ORFs of SMARCD3-OT1 (in which the termination codon TGA was mutated to TGG) were amplified and cloned into the mutational pEGFP-N1 vector (pEGFP mut).
For overexpression vector construction, the full lengths of the SMARCD3-OT1 sequence and SMARCD3X4 coding sequence (NCBI: XM_040691998.1) were cloned and inserted into the pcDNA-3.1 vector (Promega, Madison, WI, USA) by utilizing the HindIII and XhoI restriction sites.

For dual-luciferase reporter vectors construction, the SMARCD3-OT1 exon 1 (SMARCD3-OT1-E1, 1 bp–768 bp), SMARCD3-OT1 exon 2 (SMARCD3-OT1-E2, 769 bp–964 bp), SMARCD3-OT1 exon 3 (SMARCD3-OT1-E3, 965 bp–1542 bp), SMARCD3-OT1 exon 1 + 2 (SMARCD3-OT1-E1 + 2, 1 bp–1542 bp), and SMARCD3-OT (SMARCD3-OT1-E1 + 2 + 3, 1 bp–1542 bp), were cloned into the PGL3-promoter vector (Promega, Madison, WI, USA) by using the HindIII and NcoI restriction sites.

For the specific knockdown of SMARCD3-OT1 and SMARCD3X4, the ASO were designed and produced by Guangzhou RiboBio (Guangzhou, China), respectively, for SMARCD3-OT1 and SMARCD3X4 that mainly existed in the cell nucleus.

The primers pairs and oligonucleotide sequences utilized in plasmid construction and interference are offered in Tables S6 and S7.

4.6. Western Blot Analysis

The Western blot assays were performed as previously reported [46]. The antibodies and their dilutions utilized in the Western blots are listed as follows: rabbit anti-SMARCD1/3 Ab (DF10125; Affinity Biosciences, Changzhou, China; 1:1000), rabbit anti-GAPDH (AP0063; Bioworld Technology, Bloomington, MN, USA; 1:10,000), mouse anti-MyHC (B103; DHSB, USA; 0.5 µg/mL), rabbit anti-MyoD1 (P10085; Bioss, Beijing, China; 1:500), rabbit anti-MYF5 (913349; Bioss, Beijing, China; 1:500), rabbit anti-Myogenin (P15173; Bioss, Beijing, China; 1:500), GFP-tag monoclonal antibody (AP0675M; Bioworld Technology, Bloomington, MN, USA; 1:2000), and beta-actin polyclonal antibody (Ap0060; Bioworld Technology, Bloomington, MN, USA; 1:10,000). Goat Anti-rabbit IgG-HRP (BA1054; Boster, Wuhan, China; 1:10,000) and Peroxidase-goat Anti-mouse IgG (BA1051; Boster, Wuhan, China; 1:10,000) were utilized as secondary antibodies.

4.7. Immunofluorescence

Cells were cultured and seeded into a 12-well plate and were transfected for 48 h. After 48 h transfection, the CPMs were induced to differentiation for 3 days. Then, the cells were fixed by 4% formaldehyde for 30 min and were washed by phosphate-buffered saline (PBS, Gibco, Waltham, MA, USA) for 15 min. Next, the cells’ cytomembranes were destroyed by 0.1% Triton X-100 diluted by PBS for 20 min and were blocked by goat serum for 20–30 min. After that, cells were incubated with mouse anti-MyHC (B103; DHSB, Iowa City, IA, USA; 0.5 µg/mL) overnight at 4 °C, and were incubated with Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (H + L) (BS50950; Bioworld, USA; 1:50) at room temperature for 1 h. The cell nucleus was stained by DAPI (Solarbio, Beijing, China) for 10 min. A TE2000-U fluorescence microscope (Nikon, Tokyo, Japan) was utilized to capture the image of cells, and the images were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

4.8. Flow Cytometry, EdU, and CCK-8 Assays

For the analysis of cell proliferation stages, cells were cultured and seeded into a 12-well culture plate and transfected for 48 h. After pre-cooling PBS washing, cells were fixed in 70% ethanol and stored at −20 °C overnight. Then, a Cell Cycle Analysis Kit (Thermo Fisher Scientific, MA, USA) was used in flow cytometry analysis of the cell cycle. A BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) was utilized for the analysis of myoblasts. The FlowJo software (7.6, Tree Star, Ashland, OR, USA) was used for data processing.

For the EdU assay, myoblasts were seeded into a 12-well culture plate and transfected when the cells density reached 70–80%. After 48 h transfection, cells were fixed by 4% formaldehyde for 30 min and stained by using EdU Apollo In vitro Imaging Kit (C10310,
RiboBio, Guangzhou, China). A fluorescence microscope was utilized to acquire images, and ImageJ software was used for the analysis of data.

For the CCK-8 assay, primary myoblasts were seeded into 96-well culture plates and transfected. Then, the cell growth condition was monitored at 12, 24, 36, and 48 h by using the TransDetect CCK Kit (TransGen Biotech, Beijing, China) following the instruction book. The absorption spectra at 450 nm were detected by using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA). All data were acquired from six independent repeats.

4.9. Lentivirus Assay

Thirty 7-day-old chickens from Yu He Agriculture and Animal Husbandry Co., Ltd. were fed in the same room with free food as well as water and were randomly divided into two groups (n = 15): (1) LV-SMARCD3-OT1 and LV-NC, and (2) ASO-SMARCD3-OT1 and ASO-NC. Chickens were injected with lentivirus (3 × 10^6 titers) or modified ASOs (40 nmol) by intramuscular injection on days 7 and 14. The overexpressed lentivirus and ASO-SMARCD3-OT1 were injected into the chickens’ left gastrocnemius muscle, and the reagents of control groups were injected into the right gastrocnemius muscle. The chickens were euthanized at 21 days old, and the gastrocnemius muscles were detached and stored at −80 °C.

4.10. H&E Staining and Immunohistochemistry

For H&E staining, gastrocnemius muscles were fixed in 4% paraformaldehyde overnight and sent to Servicebio Co., Ltd. (Wuhan, China) for slicing up and H&E staining.

For immunohistochemistry, the sections of gastrocnemius muscle tissues were stained by using an SP-POD kit (SP0041, Solarbio, Beijing, China). The primary antibodies were anti-MYH1 (GTX17458; Genetex, Irvine, CA, USA; 1:400) and anti-MYH7 (S58; DHSB, Iowa City, IA, USA; 1:100).

4.11. Enzyme Activities Analysis

The gastrocnemius muscles induced or knockdown of SMARCD3-OT1 were collected and cut into small pieces, which were subsequently weighed and ground at 4 °C. Then, the ground samples were centrifuged, and the supernatant was collected for the subsequent detection of the activity of LDH. The LDH Activity Detection Kit (BC0685, Solarbio, Beijing, China) was used to detect the activity of LDH. The data were acquired by a fluorescence/multi-detection microplate reader (BioTek, Winooski, VT, USA) at 450 nm, and the activity of LDH was calculated based on the weight of the sample.

4.12. Dual-Luciferase Reporter Assay

The PGL3-promoter inserted with the different parts of SMARCD3-OT1 was transfected into a DF-1 cell line in a 96-well culture plate. After 48 h transfection, a Dual-Glo Luciferase Assay System Kit (Promega, Madison, WI, USA) was used to detect the firefly and Renilla luciferase activities following the manufacturer’s protocol. The data were acquired by a fluorescence/ multi-detection microplate reader (BioTek, Winooski, VT, USA). All data were acquired from eight independent repeats.

4.13. Statistical Analysis

Every assay was repeated at least three times. The data from every experiment were presented by mean ± S.E.M, and the statistical significance of differences between different groups was tested by independent or paired t tests. Independent t tests were used in cell experiments, and paired t tests were used in animal experiments. * p < 0.05 was considered as significant, and ** p < 0.01 was considered as highly significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23094510/s1.
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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of South China Agricultural University (protocol code SCAU#2020C010, 1 June 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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