Long noncoding RNA Neat1 modulates myogenesis by recruiting Ezh2

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Abstract

Neat1 is widely expressed in many tissues and cells and exerts pro-proliferation effects on many cancer cells. However, little is known about the function of Neat1 in myogenesis. Here we characterized the roles of Neat1 in muscle cell formation and muscle regeneration. Gain- or loss-of-function studies in C2C12 cells demonstrated that Neat1 accelerates myoblast proliferation but suppresses myoblast differentiation and fusion. Further, knockdown of Neat1 in vivo increased the cross-sectional area of muscle fibers but impaired muscle regeneration. Mechanically, Neat1 physically interacted with Ezh2 mainly through the core binding region (1001–1540 bp) and recruited Ezh2 to target gene promoters. Neat1 promoted myoblast proliferation mainly by decreasing the expression of the cyclin-dependent kinase inhibitor P21 gene but inhibited myoblast differentiation by suppressing the transcription of myogenic marker genes, such as Myog, Myh4, and Tnni2. Altogether, we uncover a previously unknown function of Neat1 in muscle development and the molecular mechanism by which Neat1 regulates myogenesis.

Introduction

Skeletal muscle is the most abundant tissue in the mammalian body and plays a pivotal role in regulating body metabolism and homeostasis. The differentiation of skeletal muscle cells is precisely regulated by several myogenic regulatory factors (MRFs), including myogenic differentiation 1 (Myod), myogenic factor 5 (Myf5), myogenin (Myog), and Mrf4. Myod or Myf5 is necessary for skeletal muscle lineage formation and is expressed at the myoblast stage. Myod overexpression converts fibroblasts into myoblasts and subsequent fusion into myotubes. Myog and Mrf4 are expressed after Myod and Myf5 and determine terminal muscle cell differentiation. Myog knockdown reversed terminal muscle cell differentiation. MRFs also contribute to the regeneration of injured adult muscle, as muscle regeneration depends on the activation of the muscle regulatory network during injury, satellite cells (SCs) are activated and undergoing proliferation, and paired box (Pax) 7 and Myod genes are upregulated at this stage. Next, SCs differentiate into myoblasts, upon which Pax genes are downregulated and Myog upregulated. Epigenetic regulation, such as DNA methylation, histone modifications, and noncoding RNA functions, also play important roles in the transcriptional regulation of myogenesis and ensure the normal proliferation and differentiation of muscle progenitors. Enhancer of zeste homolog 2 (Ezh2) is a subunit of the epigenetic regulator polycomb repressive complex 2 (PRC2) responsible for trimethylation of lysine 27 of histone 3, which leads to repression of gene transcription. A previous study established the important role of polycomb-mediated H3k27 methylation during myogenic differentiation. Ezh2 overexpression suppresses myogenic differentiation by silencing muscle-specific genes. Long non-coding RNAs (lncRNAs) (e.g., Linc-MD1, Lnc-mg, LncRNA-YY1, Linc-RAM, Myoline, MAR1, AK017368, AK017369, and AK017370)
SYISL, which are greater than 200 nucleotides in length and have no protein-coding capacity, were recently reported to play important roles in myogenesis by interacting with various proteins or acting as molecular sponges for miRNAs. Nuclear paraspeckle assembly transcript 1 (NEAT1, known as Neat1 in mouse) is a lncRNA that is enriched in the nucleus and essential for nuclear paraspeckle formation. Paraspeckles were recently identified as mammalian-specific nuclear bodies that are found in most cells cultured in vitro but are not essential in vivo. Paraspeckles play important roles in many gene regulation processes, such as mRNA retention, A-to-I editing, and protein sequestration. NEAT1 serves as a platform to recruit numerous paraspeckle proteins to maintain paraspeckle stability and integrity. In addition, long-range interactions among NEAT1 transcripts may exert an important architectural function in paraspeckles formation. In addition to participating in the formation of paraspeckles, NEAT1 also plays important roles in a variety of biological processes. For example, NEAT1 regulates the phenotypic switch of vascular smooth muscle cells by inhibiting SM (smooth muscle)-contractile gene expression by removing the epigenetic activator WDR5 from SM-specific gene loci. NEAT1 is widely expressed in multiple tissues and participates in the tumorigenesis of many cancers including prostate cancer, breast cancer, colorectal cancer, esophageal squamous cell carcinoma, laryngeal squamous cell cancer, and pancreatic cancer. Despite the important roles of Neat1 in regulating multiple biological processes, it is unknown whether it is involved in muscle development and regeneration. In the present study, we investigated the roles of Neat1 in myogenesis and found that Neat1 regulates myoblast proliferation and differentiation by interacting with Ezh2, defining a novel function of Neat1 in muscle development and regeneration.

Materials and methods

Cell culture

Mouse C2C12 cells were cultured in DMEM (high-glucose Dulbecco’s modified Eagle’s medium) (Hyclone, USA) containing 10% fetal bovine serum (Gibco, Australia) under moist air with 5% CO₂ at 37 °C for proliferation and in DMEM with 2% horse serum (Gibco, USA) at the same condition for differentiation.

Animals

C57 mice were purchased from Hubei center for disease control and housed in Huazhong Agricultural University under normal conditions with appropriate temperature and humidity and supplied with nutritional food and sufficient water. Animal feeding and tests were conducted based on the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Huazhong Agricultural University.

Plasmid construction, siRNA synthesis

The full-length sequence of Neat1, and coding sequences (CDS) of Ezh2 and P21 were amplified by polymerase chain reaction (PCR) with corresponding full-length or cds F/R primers using C2C12 cDNA as a template. The amplified sequences were cloned into pcDNA3.1 using T4 DNA ligase (Takara, Japan) to produce pcDNA3.1-Neat1, pcDNA3.1-Ezh2 and pcDNA3.1-P21. The truncated Neat1 were obtained by PCR using pcDNA3.1-Neat1 plasmid as a template and then were cloned into pcDNA3.1. The plasmids were confirmed by sequencing. The primers above were shown at Supplementary Table S1. siRNA oligos against mouse Neat1 (sense 5’-GGAGUCAUGCCUUAAUACAATT-3’), Ezh2 (sense 5’-GCGCAGUAGAAUGGAGAAATT-3’) and P21 (sense 5’-UGAGCAUGGCGUGAUCCCUU-3’) were designed and synthesized by GenePharma (China, Shanghai).

Transfection of plasmid, siRNA

For cell transfection, expression plasmids or siRNAs were conducted with Lipofectamine 2000 (Invitrogen, USA) as advised by the manufacturer’s protocol.

Quantitative real-time PCR

RNA samples from C2C12 cells or mice tissues were isolated using the TRIzol reagent (Invitrogen, USA). The expression of mRNA was detected by Quantitative real-time PCR (qPCR). The qPCR reaction was performed in LightCycler 480 II (Roche, Switzerland) system using SYBR®Green Real-time PCR Master Mix (Toyobo, Japan). All the experiments were designed in triplicates. The relative gene expression was calculated by the Ct (2^{ΔΔCt}) method according to the literature. The sequence primers were list at Supplementary Table S2.

Cell proliferation assays

For real-time cell proliferation monitoring assay, C2C12 cells were inoculated on a 16-well E-Plate and transfected with Neat1 expression vector or siNeat1 oligos. The cell proliferation rates were recored by the RTCA xCELLi-genec system (Roche Applied Science, Penzberg, Upper Bavaria, Germany).

For EdU staining, the EdU staining was performed using EDU kit (RiboBio, China) according to the manufacturer’s instructions. Images were captured with an Olympus IX51-A21PH fluorescence microscope (Olympus, Japan).
Cells were further analyzed by computing the percentage of EdU+ cells.

For EdU-propidium iodide (PI) flow cytometry, Edu reagent was added to C2C12 cells at a final concentration of 50 uM and incubated for 30 min at 37°C. Then the cells were harvested and fixed in 70% ethanol at 4 °C overnight. The cells were further carried out with EdU staining using EdU kit (RiboBio, China) according to the manufacturer’s instructions. After that the cells were incubated in 50 mg/ml PI for 1 h at room temperature, cells were analyzed using the FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting

C2C12 cells or mouse tissues were lysed in RIPA buffer containing 1% (v/v) phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Jiangsu, China). The western blotting was performed according to the previous publication44. The antibodies and their dilutions were shown as following: Myod (Santa Cruz Biotechnology, USA; sc-760; 1:1000), Myog (Santa Cruz Biotechnology, USA; sc-12732; 1:200), Myhc (Santa Cruz Biotechnology, USA; sc-376157; 1:3000), α-actin (Proteintech, China; 2366-1-AP; 1:1000), Tnni2 (Abcam, UK; ab184554; 1:1000), P21 (BOSTER, China; BM4382; 1:200), Ezh2 (Cell Signaling Technology, USA; 5246; 1:1000), Pcna (Servicebio, China; GB11010; 1:500), Ki67 (Abcam, UK; ab16667; 1:1000), β-actin (Santa Cruz Biotechnology, USA; sc-4777; 1:1000), Gapdh (BOSTER, China; BM3876; 1:200), Pax7 (Developmental Studies Hybridoma Bank; USA; 1:1000), eMyhc (Developmental Studies Hybridoma Bank, USA; BF-G6; 1:1000). The protein expression levels were normalized to corresponding β-actin or Gapdh and the western blotting bands signal intensities were quantified using ImageJ software.

Immunofluorescence

C2C12 cells were cultured in 24-well plate and differentiated for 2–3 days. The immunofluorescence staining was performed according to the previous publication44. The antibodies and their dilutions were shown as following: Myog (Santa Cruz Biotechnology, USA; sc-12732; 1:50) and Myhc (Santa Cruz Biotechnology, USA; sc-376157; 1:200), a secondary antibody (anti-mouse CY3; Beyotime Biotechnology, China). The 4′, 6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei. The images were visualized with a fluorescence microscope (IX51-A21PH, Olympus, Japan). The cell differentiation index was calculated by the ratio of the number of nuclei in the myotubes to the total number of nuclei in one field of view. For myoblast fusion, the cells were differentiated for 5 days. Then the cells were performed with myosin (Sigma, USA; M4276; 1:1000) immunofluorescence to test myoblast fusion. The cell fusion was calculated by the number of nuclei present in one myosin-positive cell indicated.

Knockdown of Neat1 in vivo by lentivirus infection

6-week C57 male mice were injected with 100 μl final volumes of lentivirus contained small interfere Neat1 (LV3-shNeat1) or control (LV3-shNC) at 2 × 10⁷ TU/ml into the right and left quadriceps (Qu), tibialis anterior (TA) and gastrocnemius (Gas) of the hind legs, respectively. LV3-shNeat1 or LV3-shNC was synthesized by GenePharma (China, Shanghai). LV3-shNeat1 or LV3-shNC was diluted in PBS and injected into Qu, TA and Gas of the hind legs every one week, after one month of injection, the mice were killed and the Qu, TA and Gas muscles of the hind legs were collected. For qPCR and western blotting analysis, the injection was performed in three mice. The Qu, TA and Gas muscles of the right or left hind legs of each injected mouse were collected, and used for total RNA and protein extraction. For myosin immunofluorescence, the Qu, TA and Gas muscles of the right or left hind legs of each injected mouse were fixed in 4% paraformaldehyde, respectively.

Muscle injury and regeneration

For CTX injection, 6-week male mice were injected with 100 μl final volumes of LV3-shNeat1 or LV3-shNC at 2 × 10⁷TU/ml into the right and left Gas muscles, respectively. One day after, the above mice were injected with 100ul of CTX at 80ug/ml into the Gas muscles at both hind legs. Mice were sacrificed and the Gas muscles were harvested at designed days. Mice were administered with 100 μg EdU (Thermo Fisher Scientific, Waltham, MA, USA) by intraperitoneal injection at 6 h before muscles harvesting. Extraction of the total RNAs and proteins were used for qPCR and western blotting analysis, respectively.

Histology staining

For immunofluorescence staining of Pax7, EdU, Myog and eMyhc, the Gas muscles were harvested at day 3. Immunofluorescence staining on frozen muscle sections was performed in accordance with previous reports21, and images were visualized using a confocal laser scanning microscope (Zeiss, LSM800, Germany). The following dilutions were used for each antibody: Pax7 (Developmental Studies Hybridoma Bank; USA; 1:20), eMyhc (Developmental Studies Hybridoma Bank, USA; BF-G6; 1:100), Myog (Santa Cruz Biotechnology, USA; sc-12732; 1:20). To detect the EdU incorporation, the sections were performed using the Life Technologies Click-iT Kit according to the manufacturer’s instructions, and images were photographed using a confocal laser scanning microscope (LSM800; Zeiss). For H&E staining, the Gas muscles were harvested at day 0, 3, 7, and 15 after CTX injection. H&E of muscle sections was performed according to previous reported methods21,45, and the cross-section area of individual myofibers was visualized using Olympus DP80 upright.
Metallurgical Microscope (Olympus Corporation, Japan) and qualified using ImageJ software.

RNA immunoprecipitation assay
RNA immunoprecipitation (RIP) assays were conducted using EZ-Magna RIP Kit (Millipore) according to the manufacturer's instructions. Briefly, C2C12 cells were lysed with RIP lysis buffer, and incubated with 1 μg antibody (Ezh2, Abcam, ab3748; Suz12, Abcam, ab12073; IgG, Millipore) for RNA immunoprecipitation at 4 °C overnight. Then Protein A/G beads were added to the lysates to pull down antibody-protein-RNA complex. The detection of co-precipitated RNAs was performed by reverse-transcription polymerase chain reaction (RT-PCR).

RNA pulldown assays
RNA pulldown was performed as previously described46. Briefly, linearizing DNA was biotin-labeled and in vitro transcribed using the Biotin RNA Labeling Mix and T7/SP6 RNA polymerase (Roche), and purified with the RNeasy Mini Kit (QIAGEN). One milligram of protein was incubated with 3 μg of biotinylated RNA for 1 h at room temperature. After that, 40μl Streptavidin-coupled Dynabeads (Invitrogen) were added to each reaction and incubated for 1 h at room temperature. Finally, the beads were washed in RIP buffer for five times, and the pull-downed proteins were used for western blotting. For mass spectrometry, the pull-downed proteins in C2C12 cells were separated by 10% SDS-PAGE, and then performed with silver staining. The differentially expressed bands were excised and analyzed by mass spectrometry (Novogene, Beijing, China).

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assay was performed using ChIP Kit (Millipore, 17–371) according to the manufacturer’s instructions. Each ChIP reaction was performed using 1 μg of antibodies against Ezh2 (Abcam, ab3748), H3k27me3 (Abcam, ab6002) or IgG applied as negative control. Fold enrichment was quantified using qPCR. All promoter primers were listed in Supplementary Table S3.

Chromatin Isolation by RNA Purification assays
Chromatin Isolation by RNA Purification (ChIRP) assay was performed using ChIRP Kit (Millipore#17–10495) according to the manufacturer’s instructions. Biotin-labeled Neat1 probes were designed by Guangzhou Ribobio and divided into odd and even pools. C2C12 cells were cross-linked with 1% glutaraldehyde and lysed with lysis buffer before sonication for 4 h. Odd and even Neat1 probes were added into samples for incubation at 37 °C for 4.5 h with rotating. The combined chromatin fragments were enriched by C-1 streptavidin beads and purified for qPCR experiment.

Statistical analysis
All data were expressed as mean ± standard deviation (s.d.). Statistical analyses between different groups were performed using t-test. For all analyses, p < 0.05 was considered to be statistically significant.

Results
Neat1 is up-regulated during myogenic differentiation and muscle regeneration
To investigate the role of Neat1 in myogenesis, we first examined the expression profiles of Neat1 as well as Myog and Myhc during myogenic differentiation. The expression levels of Neat1 and Myog were increased in C2C12 cells from day 0 (proliferating cells) to day 5 post differentiation but decreased on day 8, whereas Myhc expression gradually increased during differentiation (Fig. 1a–c).

To further explore Neat1 expression during muscle regeneration, we employed an extensively used muscle regeneration model in which intramuscular injection of cardiotoxin (CTX) leads to muscle injury and induces muscle regeneration47. The expression levels of Neat1 along with Pax7 and Myod were highly increased during the early stage of regeneration and then decreased when the newly formed fibers maturation and regeneration completed. We also found that Neat1 expression peaked earlier than that of Pax7 and Myod (Fig. 1d–f). These results suggest that Neat1 is involved in myogenesis and muscle injury repair.

Neat1 promotes myoblast proliferation, but inhibits myogenic differentiation and fusion
NEAT1 promotes tumor growth in many cancer cells, including prostate cancer37, breast cancer cell lines38, colorectal cancer39. To investigate the roles of Neat1 in myoblast proliferation, we conducted Neat1 knockdown and overexpression experiments in C2C12 cells. Neat1 knockdown led to a significant reduction in Ki67 and Pcnna mRNA expression and Ki67 protein expression (Fig. 2a, b), whereas Neat1 overexpression had the opposite effect (Supplementary Fig. 1a–b). RTCA xCEL-Ligence, EdU-PI flow cytometry assays confirmed the effects of Neat1 on cell proliferation. The RTCA xCEL-Ligence assay suggested that Neat1 knockdown significantly reduced cell growth (Fig. 2c), while Neat1 overexpression enhanced cell growth (Supplementary Fig. 1c). EdU-PI flow cytometry assays showed a significant decrease and increase in DNA replication (S-phase) after Neat1 knockdown and overexpression, respectively (Fig. 2d and Supplementary Fig. 1d–e). These observations indicate that Neat1 promotes the proliferation of C2C12 cells.

To verify the functions of Neat1 during C2C12 differentiation, Neat1 was knocked down and overexpressed during C2C12 cell differentiation. Knockdown of Neat1
increased the mRNA expression of myogenic marker genes, such as Myod, Myog, Myhc, α-actin, and Tnni2 (Fig. 2e). Neat1 knockdown also enhanced myogenic differentiation, as indicated by a significant increase in the protein expression of these genes during cell differentiation (Fig. 2f and Supplementary Fig. 2a). Immunofluorescence staining of Myog and Myhc revealed an increased number of Myog+ and Myhc+ cells (Fig. 2g, h). These observations were also confirmed by Neat1 overexpression (Supplementary Fig. 2b-f). Together, these data demonstrate that Neat1 inhibits myoblast differentiation.

Finally, to investigate whether Neat1 affects myoblasts fusion, myosin immunofluorescence staining was used to analyze myoblasts fusion after Neat1 knockdown or overexpression. The results showed that Neat1 knockdown increased the ratio of myotubes with more than three nuclei, while Neat1 overexpression decreased the ratio of myotubes with more than three nuclei (Fig. 2i and Supplementary Fig. 2g). In addition, Neat1 knockdown and overexpression significantly increased and decreased the fusion marker gene Myomaker expression, respectively (Supplementary Fig. 2h–i), suggesting Neat1 inhibits myoblasts fusion.

**Knockdown of Neat1 promotes postnatal muscle growth in vivo**

To investigate the effects of Neat1 on muscle growth in vivo, 6-week-old C57 mice were injected with LV3-shNeat1 or LV3-shNC particles into the right and left hindlimbs, respectively. The injection scheme is shown in Fig. 3a. The injection of LV3-shNeat1 particles led to a significant reduction of Neat1 expression (Fig. 3b). The mRNA and protein levels of Myog, Myhc, Tnni2, and α-actin genes were significantly increased after Neat1 knockdown (Fig. 3b, c). Further, the volume and weight of the Qu, TA, and Gas muscles in Neat1 knockdown groups were larger than those of the controls (Fig. 3d, e). Immunofluorescence staining for myosin showed that the cross-sectional areas of the Qu, TA, and Gas muscles injected with LV3-shNeat1 particles were dramatically larger than those injected with LV3-shNC particles (Fig. 3f). These results indicate that Neat1 knockdown enhances muscle growth.

**Neat1 knockdown delays muscle regeneration after CTX injection in vivo**

To determine whether Neat1 regulates CTX-induced muscle regeneration, 6-week-old C57 mice were injected with LV3-shNeat1 or LV3-shNC particles into the Gas muscles of the right and left hindlimbs, respectively, followed by CTX injection (Fig. 4a). The muscle regeneration phenotype was evaluated by H&E staining of muscle sections on days 0, 1, 3, 7, and 15 after CTX treatment. On day 7 post-CTX treatment, the LV3-shNeat1 injection group displayed more inflammatory cells and fewer newly formed myofibers. On day 15 post-CTX treatment, the newly formed myofibers with the central nucleus were smaller in the LV3-shNeat1 injection group than those of the LV3-shNC injection group (Fig. 4b), and the
percentage of fibers with the central nucleus was higher in LV3-shNeat1 group than that in LV3-shNC groups (Fig. 4c). Knockdown of Neat1 also led to a decrease in both the mRNA and protein levels of Pax7, Myod, Myog, and eMyhc genes, as well as the Ki67 mRNA level, on day 7 post-CTX injection (Fig. 4d, e). Immunofluorescence staining in muscle sections showed that the percentage of both Pax7+ SCs and proliferating Pax7 (Pax7+/EdU+) SCs were reduced in the LV3-shNeat1 injection group compared with the controls on day 3 post-CTX injection (Fig. 4f). Immunofluorescence staining in muscle sections also showed that knockdown of Neat1 decreased the number
of cells with positive staining of Myog and eMyhc on day 3 post-CTX injection (Fig. 4g, h). Reduced Myog and eMyhc expression in the LV3-shNeat1 injection groups is likely the consequence of impaired SC proliferation rather than impaired differentiation, as Neat1 knockdown promoted Myog and Myhc expression. Together, these results suggest that Neat1 knockdown delays muscle regeneration following CTX treatment.

Neat1 physically interacts with Ezh2

As a well-known nuclear lncRNA, Neat1 may play a role at the transcriptional level. A recent study revealed that NEAT1 interacts with EZH2 in human glioblastoma cells48. Our previous studies showed that Ezh2 plays important roles in myoblast proliferation and differentiation by increasing the level of H3k27me3 binding at gene promoters27; therefore, we inferred that Neat1 may also regulate myoblast proliferation and myogenic differentiation by interacting with Ezh2. First, we performed RNA immunoprecipitation (RIP) assays to confirm the interaction between Neat1 and Ezh2, and as expected, Neat1 was significantly enriched on the Ezh2 antibody compared with the IgG antibody (negative control) (Fig. 5a). To confirm the interaction, biotin-labeled full-length Neat1 was used to pull down target proteins. RNA pull-down assays revealed that Neat1 transcripts pulled down endogenous Ezh2 but not Myod or Pcna (Fig. 5b), and there was no mutual effect between Neat1 and Ezh2 expression (Supplementary Fig. 3a–c), suggesting that Neat1 physically binds to Ezh2. As PRC2 contains several members, such as Ezh2 and Suz12, we also evaluated the interaction between Neat1 and Suz12. As expected, Neat1 was also pulled down by the Suz12 antibody (Supplementary Fig. 3d). Next, a series of deletions in full-length Neat1 was performed to determine the core binding domain (Fig. 5c). Interestingly, the third
fragment (1001–1540, F3) was efficiently pulled down Ezh2, whereas fragments F1, F2, and F4 rarely pulled down Ezh2 (Fig. 5c). To further verify the core functional domain of Neat1, F1, F2, F3, F4, and full-length Neat1 were overexpressed in C2C12 cells. EdU staining showed that the overexpression of F3 and full-length Neat1 significantly increased EdU incorporation compared with the control (Fig. 5d and Supplementary Fig. 3e).
Immunofluorescence staining also revealed a significantly decreased number of Myog$^+$ and Myhc$^+$ cells when F3 and full-length Neat1 were overexpressed (Fig. 5e, f and Supplementary Fig. 3f–g). These results indicate that the F3 fragment of Neat1 is required for its recruitment of Ezh2 in myogenesis. Besides, we also identified 122 Neat1-binding proteins in C2C12 cells by mass spectrometry including some known interacting paraseque proteins (Sfpq, Hnrnpq, Hnrnpm and Matr3) and some proteins involved in muscle development and disease (Eef2, Dnm2, Trim28, Trpv2, Wfs1, Pml, Vcp, Lmna and Matr3) (Supplementary Fig. 3h and Supplementary Table 4).

**Neat1 enhances the proliferation of C2C12 cells via Ezh2-mediated H3k27me3 enrichment at the P21 promoter**

Previous studies have shown that IncRNAs such as SNHG2059, Xist50 and SYSL27 repress the cyclin-dependent kinase inhibitor 1 A (P21) by interacting with Ezh2, as well as promote cell proliferation. Our above-mentioned results showed that Neat1 promotes proliferation and interacts with Ezh2 in C2C12 cells (Fig. 2a–d and Supplementary Fig. 1 and Fig. 5). Further, the overexpression of P21 significantly reduced the number of EdU$^+$ cells, whereas Ezh2 overexpression increased the number of EdU$^+$ cells (Supplementary Fig. 4a-b), demonstrating that P21 and Ezh2 have the opposite effects on C2C12 cell proliferation. Thus, we inferred that Neat1 facilitates C2C12 cell proliferation by Ezh2-mediated inhibition of P21 expression. First, we explored whether Neat1 regulates P21 expression and found that knockdown of Neat1 inhibited cell cycle-dependent kinase 4 (Cdk4) and enhanced P21 protein expression, indicating that Neat1 inhibits P21 expression in C2C12 cells (Fig. 6a). A previous study showed that Ezh2 inhibits P21 expression by directly binding to its promoter27. Therefore, we performed ChIP-qPCR assays to explore whether Neat1 affects the Ezh2- and H3k27me3-binding capacities at the P21 promoter. Knockdown and overexpression of Neat1 decreased and increased the enrichment of Ezh2 and H3k27me3 at the P21 promoter, respectively (Fig. 6b–e). To further elucidate whether Neat1 affected P21 expression through Ezh2, Ezh2 siRNA fragment and Neat1 expression vector were co-transfected into C2C12 cells. Immunofluorescence staining of P21 revealed that Neat1 overexpression inhibited P21 expression, but had no effect when co-transfected with Ezh2 siRNA fragments (Fig. 6f and Supplementary Fig. 4c). We also co-transfected Neat1 siRNA fragments with Ezh2 expression vector in C2C12 cells and assessed cell proliferation by EdU staining. Knockdown of Neat1 significantly reduced the percentage of EdU$^+$ cells. After transfection with Ezh2 expression vector, Neat1 knockdown did not reduce the number of EdU$^+$ cells (Fig. 6g and Supplementary Fig. 4d), indicating that Neat1 regulation of myoblast proliferation is dependent on Ezh2. To further confirm whether Neat1 promoted cell proliferation through P21 pathway, Neat1 and P21 siRNA fragments were co-transfected into C2C12 cells, and then the cell proliferation ability was detected by EdU staining. The results showed that Neat1 knockdown significantly reduced the percentage of EdU$^+$ cells, but had no effect after co-transfection with P21 siRNA fragment (Fig. 6h and Supplementary Fig. 4e). Together, these results suggest that Neat1 inhibits P21 expression by increasing the Ezh2-binding capacities at its promoter, thereby promoting C2C12 cell proliferation.

**Neat1 inhibits myogenic differentiation by epigenetically silencing the expression of myogenic markers**

Previous studies have shown that Ezh2 suppresses myogenic differentiation by increasing levels of the epigenetic silencing marker H3k27me3 to repress the transcription of myogenic markers such as Myog$^{18,51}$,
myh4, Tnni2, and Tnni2. Therefore, we performed ChIP assays to determine whether Neat1 affects the inhibitory effects of Ezh2 on the expression of these myogenic genes. ChIP-qPCR assays suggested that knockdown of Neat1 decreased the binding of Ezh2 and H3k27me3 at the Myog, Myh4, and Tnni2 gene promoters (Fig. 7a, b), which was confirmed by Neat1 overexpression (Fig. 7c, d). To further elucidate whether Neat1 affects these target genes via Ezh2, Neat1 expression vector and Ezh2 siRNA fragment were co-transfected into C2C12 cells. As expected, the overexpression of Neat1 remarkably reduced the expression of Myog, Myhc, and Tnni2, but not when co-transfected with the Ezh2 siRNA fragments (Fig. 7e and Supplementary Fig. 5a–b). Immunofluorescence staining of Myhc also showed that Neat1 knockdown enhanced Myhc expression, but not when co-transfected with Ezh2 expression vector (Fig. 7f and Supplementary Fig. 5c).

Lastly, we performed chromatin isolation by RNA purification (ChIRP) to confirm that Neat1 binds directly to the Myog, Myh4, Tnni2, and P21 gene promoters but not to the Myod promoter in C2C12 cells and mouse primary myoblasts (Fig. 7g, h), consistent with the pattern

![Fig. 5 Neat1 interacts with Ezh2.](image)
of Ezh2 occupancy at its target genes (Fig. 6b–d and Fig. 7a, c and Supplementary Fig. 5d), indicating Neat1 regulated Myod expression independent of Ezh2. Altogether, these results suggest that Neat1 suppresses C2C12 myogenic differentiation mainly by increasing Ezh2 enrichment at the promoters of target genes.

**Discussion**

*NEAT1* is involved in multiple biological processes in vitro and in vivo. In vitro, *NEAT1* affects the proliferation, migration, invasion, and apoptosis of multiple cancer cells; for example, *NEAT1* promotes the proliferation and invasion of colorectal cancer cells.
Knockdown of NEAT1 suppresses the migration and invasion of glioma cells\textsuperscript{55}. NEAT1 is regulated by c-myc and inhibits imatinib-induced apoptosis of chronic myeloid leukemia cells\textsuperscript{35}. Besides, NEAT1 also plays an important role in vascular smooth muscle cell phenotypic switching\textsuperscript{36}. In vivo, NEAT1 is overexpressed in many
solid tumors, including small cell lung cancer \(^{67}\) and hepatocellular carcinoma \(^{58,59}\). Neat1 knockout mice display impaired corpus luteum differentiation \(^{60}\). The genetic ablation of Neat1 leads to abnormal mammary gland morphogenesis and lactation defects \(^{61}\). Neat1 exerts anti-apoptotic and anti-inflammatory functions in C57BL/6 mice after traumatic brain injury \(^{62}\). The functions of Neat1 in myogenesis and skeletal muscle development remain unexplored. Here, we demonstrated that Neat1 promotes myoblast proliferation and inhibits myogenic differentiation. Moreover, knockdown of Neat1 improved the cross-sectional area of muscle fibers, mainly by increasing the expression of myogenic genes, and delaying muscle regeneration, primarily via a reduction in the number of Pax7 \(^+\) cells. In general, our study found a previously unidentified function of Neat1 in regulating muscle development and regeneration.

LncRNAs regulate gene expression at the transcriptional and post-transcriptional levels or by chromatin modifications \(^{63–65}\). As a well-known nuclear lncRNA, NEAT1 functions mainly as a transcriptional regulator. Capture hybridization analysis of RNA targets (CHART) analysis revealed that NEAT1 binds directly to both the transcriptional start sites and transcriptional termination sites of target genes. CHART-mass spectrometry assays identified a large number of NEAT1-interacting proteins \(^{66}\), suggesting that the function of NEAT1 in transcriptional regulation may be mediated by many proteins. In the present study, we found that Neat1 promoted myoblast proliferation and inhibited myogenic differentiation by guiding Ezh2 to target gene promoters, such as Myog, Myh4, Tnni2, and P21, and repressed their transcription. Ezh2 is an important epigenetic inhibitory factor involved in many biological processes, including myogenesis. Ezh2 suppresses myogenic differentiation mainly by inhibiting the expression of myogenic marker genes, such as Myog, myh4, and Mck \(^{18,51,52}\). A previous study showed that the conditional knockout of Ezh2 in SCs resulted in decreased muscle regeneration and number of Pax7 \(^+\) cells \(^{67}\), consistent with the phenotype of Neat1 knockdown. Moreover, Neat1 knockdown in vivo increased the expression of myogenic genes and the myofiber cross-sectional area, which may be also mediated by Ezh2, because Ezh2 muscle-specific knock out SCs also enhanced myogenic differentiation reflected by increased Myog expression \(^{67}\). Therefore, we conclude that the Neat1 is an important regulator of Ezh2-mediated epigenetic regulation in myogenesis and muscle regeneration. In addition to interacting with Ezh2, Neat1 may regulate myogenesis via other proteins or signaling pathways, because Neat1 also inhibited Myod expression in an Ezh2-independent manner. Further endeavors will be devoted to determining the other mechanisms by which Neat1 affects myogenesis.

LncRNAs are highly conserved in terms of their position within the genome, and conserved lncRNAs may play similar functions among species. For example, linc-YY1 and lincMyoD are both conserved in their genomic positions and are involved in myogenesis in both mouse and human \(^{68,69}\). Compared with protein-coding genes, lncRNAs have low sequence conservation. However, some lncRNAs that possess “ultraconserved” regions also have conserved functions \(^{70,71}\). For example, the lncRNA THOR exerts a carcinogenic role by interacting with IGF2BP1 via an ultraconserved region in human, mouse, and zebrafish \(^{72}\). In the current study, we observed that the F3 region (1001–1540 bp) of Neat1 encompasses the functional domain due to its interaction with Ezh2 and regulation of myogenesis, and further sequence alignment analysis demonstrated that this 1001–1540 bp region is also conserved between mouse and human (Supplementary Fig. 6), indicating a potentially ultraconserved region. In addition, a previous study showed that lncRNA higher-order structures are highly conserved, and these conserved secondary and tertiary structures are related to their biological functions and protein-binding potential \(^{73,74}\). Therefore, we speculated that this 1001–1540 bp region of Neat1 may also contribute to the formation of Neat1 higher-order structures to allow binding to its interacting proteins, and Neat1 may have a conserved function in myogenesis between human and mouse.

In conclusion, our findings provide a novel function of Neat1 in muscle development and regeneration. We demonstrated that Neat1 promoted myoblast proliferation and repressed myogenic differentiation, and knockdown of Neat1 promoted muscle growth but impaired...
muscle regeneration. Mechanistically, Neat1 recruited Ezh2 to increase the level of H3k27me3 binding at the P21 promoter, leading to repression of P21 expression and promotion of myoblast proliferation. Meanwhile, Neat1 inhibited the expression of muscle-specific genes, such as Myog, Myh4, and Tnni2, by recruiting Ezh2 to target gene promoters and thereby suppressing myogenic differentiation (Fig. 8).

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Conflict of interest
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