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# siRNA knockdown of alanine aminopeptidase impairs myoblast proliferation and differentiation

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ABSTRACT

A large number of intracellular proteins are degraded by the ubiquitin-proteasome system, one of the major protein degradation pathways. It produces peptides of several different sizes through protein degradation, and these peptides are rapidly degraded into free amino acids by various intracellular aminopeptidases. Previously, we reported that the activity of proteasomes and aminopeptidases in the proteolysis pathway are necessary for myoblast proliferation and differentiation. However, the detailed function of intracellular aminopeptidases in myoblast proliferation and differentiation has not yet been elucidated. In this study, we focused on alanine aminopeptidase (APN) and investigated the function of APN in C2C12 myoblast proliferation and differentiation. In myoblasts and myotubes, APN was mainly localized in the cell membrane as well as expressed at low levels in the cytoplasm and nucleus. The reduction of the APN enzymatic activity impaired the cell cycle progression in C2C12 myoblasts. In addition, apoptosis was induced after APN-knockdown. Finally, myogenic differentiation was also delayed in the APN-suppressed myoblasts. These findings indicate that APN is required for myoblast proliferation and differentiation.

## 1. Introduction

Skeletal muscle formation occurs during several processes, including embryonic development and the growth and regeneration of adult skeletal muscle. The formation of skeletal muscle depends primarily on muscle stem cells, also known as satellite cells, which provide the myoblasts required for muscle regeneration and growth [1,2]. Upon activation, satellite cells proliferate as myoblasts; fusion of these myoblasts forms multinucleated myotubes, which contribute to skeletal muscle formation [3]. This complex process is controlled by the expression of myogenic regulatory factors, including transcription factors such as Myf5, MyoD, and myogenin [4].

The ubiquitin–proteasome system is a major intracellular protein degradation pathway [5,6]. Previously, we demonstrated that proteasomal dysfunction in skeletal muscle resulted in significant impairments in muscle growth and development [7]. Furthermore, we demonstrated that proteasomal dysfunction in satellite cell-specific led to the loss of the satellite cell pool, which critically compromised the regenerative capacity of skeletal muscle tissue after cardiotoxin-induced damage. Primary myoblasts derived from proteasome-defective mice exhibited significantly impaired proliferation and differentiation abilities [8]. Together, these findings suggest that proteasome function is

*Abbreviations:* APN, Alanyl aminopeptidase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; KD, Knockdown; p-Rb, Phosphorylation-retinoblastoma; CCND1, Cyclin D1; CCND3, Cyclin D3; CDK4, Cyclin-Dependent Kinase 4; p21, Cyclin-dependent kinase inhibitor 1; C-Cas3, Cleaved Caspase-3; CBB, Coomassie Brilliant Blue; MyoD, Myogenic Differentiation 1; MyHC, Myosin Heavy Chain.

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indispensable for myogenesis and the maintenance of skeletal muscle tissues. The mechanism responsible for impairments in proliferation and differentiation may therefore be dependent on processes of protein degradation.

Aminopeptidases are enzymes that selectively hydrolyze amino acid residues at the N-terminuses of proteins and peptides [9,10]. Recently, we demonstrated that intracellular aminopeptidase inhibition by Bestatin methyl ester (Bes-ME) suppressed alanine aminopeptidase enzyme activity and impaired myogenesis, including proliferation and differentiation ability, in C2C12 myoblasts [11]. Based on these results, alanine aminopeptidase (APN; a protein expressed on the surface of cell membranes and is also called CD13 because it was discovered as a marker protein for stem cells) might contributes to aspects of myogenesis such as myoblast proliferation and differentiation. APN is an aminopeptidase [12] that has been identified as a marker in human embryonic stem cells and mesenchymal stem cells [13,14]. In skeletal muscle, APN is expressed in satellite cells and may contribute to several of the functions of these cells, including adhesion, cell migration, and proliferation. Muscle regeneration capacity after ischemic injury was shown to be impaired in APN-null mice [15,16]. However, little is known about the role of APN in myoblast proliferation and differentiation during myogenesis. In the present study, therefore, we investigated whether APN knockdown (KD) impairs the proliferation and differentiation ability of C2C12 myoblasts.

## 2. Material and methods

### 2.1. Cell culture

Mouse C2C12 myoblasts were cultured under the standard conditions of 37 °C and a 5% $CO_2$ humidified atmosphere in high-glucose Dulbecco's Modified Eagle Medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) that was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 100 mg/mL penicillin-streptomycin solution (Sigma-Aldrich Corporation, St. Louis, MO, USA). Myogenic differentiation was induced in DMEM supplemented with 2% calf serum (Thermo Fisher Scientific) and 100 mg/mL penicillin-streptomycin. Most myoblasts began to fuse after 1 or 2 days and formed mature myotubes by 5 days. MyoD is one of the earliest markers of myogenic differentiation, and myogenin is a later marker of myogenic differentiation. MyHC is the motor protein of muscle thick filaments [4]. For cell counting, we prepared a 1:1 dilution of the cell suspension with 0.4% trypan blue solution (Nacalai Tesque, Kyoto, Japan). The cells were counted in four 1-$mm^2$-square areas with a hemocytometer to determine the average cell numbers.

### 2.2. RNA interference

Two small-interfering RNA (siRNA) sequences targeting APN were used: i) siRNA#1 corresponding to nucleotides 2563–2583 of the APN open-reading frame and ii) siRNA#2 corresponding to nucleotides 678–698 (Sigma-Aldrich Corporation). A scramble sequence of siRNA was used as the negative control. For the siRNA knock-down experiments, C2C12 myoblasts were cultured in 6-, 12-, or 24-well plates ($5 \times 10^4$ cells, $1 \times 10^4$ cells, $0.5 \times 10^4$ cells, respectively). The cells in each well were transfected with 20 nM APN or scramble siRNAs using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, CA, USA) for 1 day or 2 days, as per the manufacturer's instruction.

### 2.3. Quantitative real-time PCR (qPCR)

Total RNA was extracted from the cultured cells by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and then reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time qPCR was performed using the StepOnePlus PCR System with the SYBR Green Master Mix (Thermo Fisher Scientific). The primer sequences for C2C12 were as follows: APN forward, GAGCAGCCTCAACTACTTCACA; APN reverse, TCATTGTACTGCTCCATCAGCG; GAPDH forward, AACTTTGGCATTGTGGAAGG; GAPDH reverse, CACATTGGGGGTAGGAACAC.

### 2.4. Western blotting

Protein lysate was obtained from the cultured cells in the RIPA lysis buffer using a protease inhibitor and a phosphatase inhibitor (ATTO Corporation, Tokyo, Japan). We performed the bicinchoninic acid (BCA) assay to determine the protein concentrations. Next, we extracted the protein fractions with a reducing sample buffer containing 7% β-mercaptoethanol. The protein extracts (10–20 μg/lane) were separated on a 5–20%-gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo System (Bio-Rad Laboratories, Inc., CA, USA). The PVDF membrane was blocked with 3% bovine serum albumins (Sigma-Aldrich) in 1X tris-buffered saline (TBS)–1% Tween-20 (FUJIFILM Wako Pure Chemical Corporation) for 1 h at room temperature. The PVDF membrane was incubated with a primary antibody for 1 h at room temperature or overnight at 4 °C with continuous shaking. The primary antibodies and dilution factors used were as follows: anti-APN (1:500; abcam, Cambridge, UK), anti-Phospho-Rb (Retinoblastoma) (1:500; Cell Signaling Technology), anti-Rb (1:500; Cell Signaling Technology), anti-Cyclin D1 (1:3000; Proteintech, IL, USA), anti-Cyclin D3 (1:1000; Cell Signaling Technology, Inc., MA, USA), anti-CDK4 (1:1000; Proteintech), anti-p21 (1:1000; Proteintech), anti-Cleaved Caspase-3 (1:1000; Cell Signaling Technology), anti-MyHC (1:1000; Santa Cruz Biotechnology, TX, USA), anti-Myogenin (1:500; Santa Cruz Biotechnology), anti-MyoD (1:500; Santa Cruz Biotechnology), and anti-GAPDH (1:2000; Cell Signaling Technology). After repeated washing, the PVDF membrane was incubated in 2% nonfat milk containing a horseradish peroxidase-conjugated secondary antibody for 1 h. The resultant bands were visualized using enhanced chemiluminescence reagents (GE Healthcare, IL, USA). Densitometry was performed using Image Lab software (Bio-Rad Laboratories).

### 2.5. Immunocytochemistry

Immunocytochemistry of C2C12 myoblasts and myotube was performed as described previously [11]. All samples were fixed by 4% Paraformaldehyde Phosphate Buffer Solution (PFA; FUJIFILM Wako Pure Chemical Corporation) for 10 min at room temperature. Permeabilization samples were incubated with primary antibodies at 4 °C overnight following blocking/permeabilization with phosphate-buffered saline containing 0.1% Triton X-100 and 5% goat serum for 60 min at room temperature. Non-Permeabilization samples were incubated with primary antibodies at 4 °C overnight following blocking with phosphate-buffered saline containing 5% goat serum for 60 min at room temperature. Immunostaining of APN (1:100; Proteintech), anti-Ki67 (1:500; abcam), anti-MyHC (1:200; Santa Cruz Biotechnology), and nuclei were visualized using appropriate species-specific Alexa Fluor-488 or Alexa Fluor-555 fluorescence-conjugated secondary antibodies and Hoechst33342 (Thermo Fisher Scientific). Staining of the cell membrane and nuclei were visualized using Acti-stain™ 555 Fluorescent Phalloidin (1:2000; Cytoskeleton, Inc., CO, USA) and Hoechst33342 (Thermo Fisher Scientific). Samples were then examined using an Olympus fluorescence microscope (Olympus Corporation, Tokyo, Japan). The relative ratio of Ki67-positive cells was calculated by dividing the number of Ki67-positive cells by the total number of nuclei namely the total number of cells. The differentiation index was calculated by dividing the number of nuclei in myotubes (MyHC-positive elongated cells) by the total number of nuclei. The length of the myotube were measured by the ImageJ Fiji software [17].

### 2.6. Aminopeptidase enzymatic activity

C2C12 myoblasts were cultured for 2 days after siRNA treatment. Additionally, as a negative sample in which APN activity was inhibited by Bes-ME (100 μM) was prepared. Cells were disrupted with RIPA lysis buffer and measured protein concentration using Qubit (Thermo Fisher Scientific) for normalization. The lysate was combined with 0.7 mM alanine substrate (L-alanine-*p*-nitroaniline; Peptide Institute, Inc., Osaka, Japan) for 2 h at 37 °C. Enzymatic activity was measured using a Multiskan Go Microplate Reader (Thermo Fisher Scientific) at an

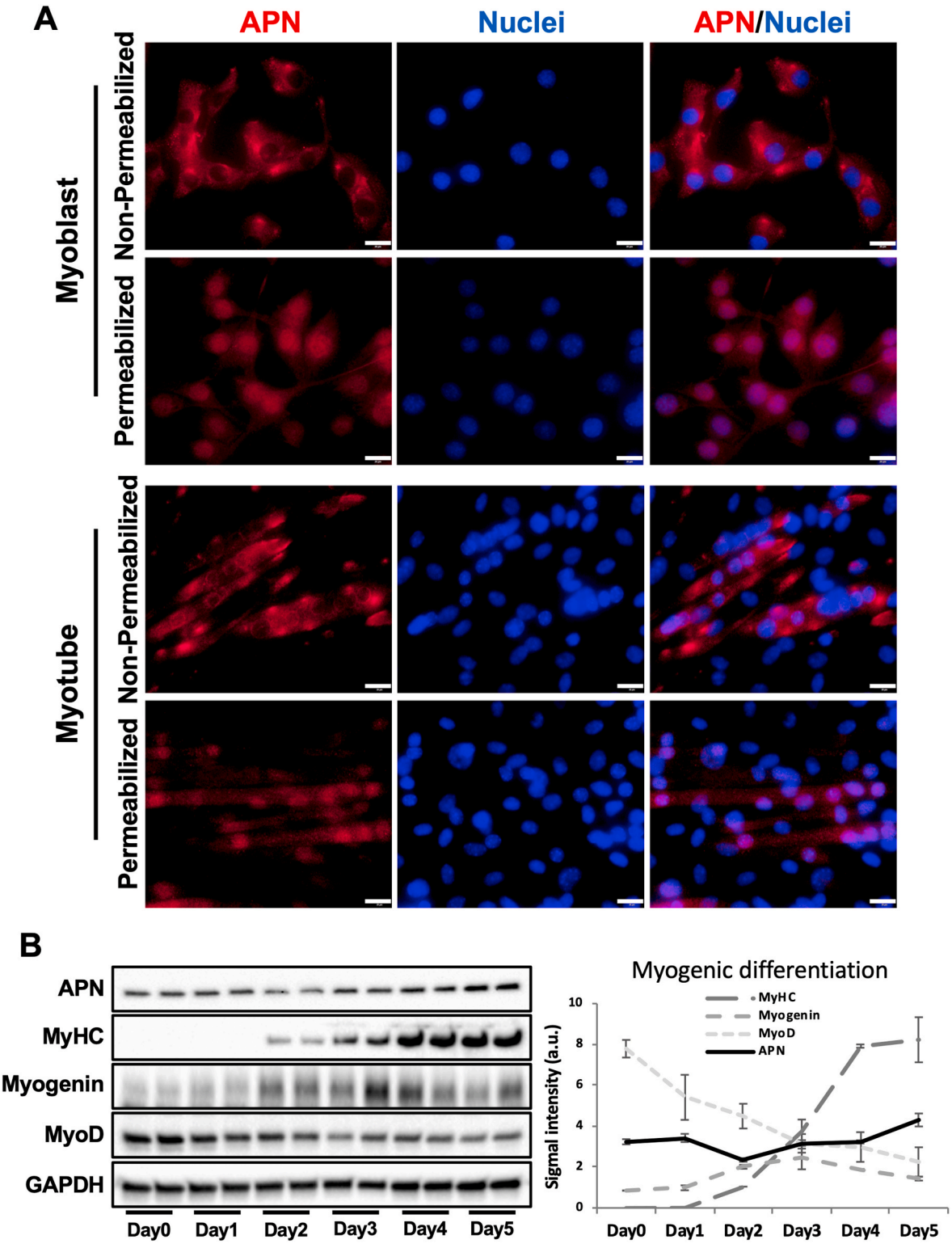


**Fig. 1.** Localization and expression of alanine aminopeptidase (APN) in myoblasts and myotubes. (A) Immunocytochemistry for APN (red) and nuclei (blue) in myoblasts and myotubes. Permeabilized means the stain of cytoplasm and nuclei. Non-Permeabilized means the stain of the cell membrane. APN mainly localized in the cell membrane. Scale bar: 20 μm. (B) Immunoblotting analysis of protein levels of APN, MyHC, Myogenin, MyoD, and GAPDH during myogenic differentiation. The expression levels of APN was slightly increased during myogenic differentiation.

excitation and emission wavelength of 405 nm, normalized by protein concentration [18].

### 2.7. Cell damage assay

Cell damage level was measured using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instruction. The C2C12 myoblasts were cultured for 2 days after siRNA treatment in 12 well plates. 100 μL of the cultured supernatant was transferred to a new 96-well plate, 100 μL of the working solution was added to the wells, and incubated for 60 min. Finally, 50 μL of the stop solution was added to the wells, and the absorbance at 490 nm was measured using a Multiskan Go Microplate Reader (Thermo Fisher Scientific).

### 2.8. Statistical analyses

Statistical analyses were performed using JMP Pro 15 (SAS Institute, Cary, NC, USA) to determine significant differences from a two-tailed distribution using Student's *t*-test. $P < 0.05$ denoted statistical significance. Data are presented as the mean ± SEM.

## 3. Results

### 3.1. Localization and expression of APN in myoblasts and myotubes

First, to identify the localization of APN in myoblasts and myotubes, we performed immunocytochemistry in both non-permeabilized and permeabilized cells. We found that in myoblasts and myotubes, APN was mainly localized in the cell membrane, but was also expressed at low levels in the cytoplasm and nucleus (Fig. 1A). Next, we examined changes in the expression of APN during muscle differentiation. The expression level of APN gradually increased with myogenic differentiation (Fig. 1B).

### 3.2. Enzymatic activity is suppressed in APN-knockdown myoblasts

To inhibit APN mRNA and protein expression, APN gene knockdown was performed in C2C12 myoblasts using two siRNAs. siRNA#1 reduced APN gene expression by more than 95% in myoblasts compared with scrambled siRNA (Control; Fig. 2A). APN protein levels were significantly reduced in cells transfected with siRNA#1 (Fig. 2B). Therefore, we decided to use siRNA#1 for APN suppression in subsequent experiments. Alanine aminopeptidase specific inhibition by Bes-ME treatment markedly inhibited to the enzymatic activity of alanine substrates (Fig. 2C). Similarly, in APN-KD myoblasts, the enzymatic activity of APN against an alanine substrate was significantly suppressed (Fig. 2C).

### 3.3. APN-knockdown impairs cell cycle progression and induces apoptosis

To investigate the effect of APN-knockdown on cell proliferation ability in myoblasts, we performed cell counts, western blotting analysis, and immunocytochemistry (Fig. 3A). The number of cells significantly decreased and cell morphology changed in slender shape after 2 d of siRNA treatment in APN-KD myoblasts (Fig. 3B). Subsequently, we examined the expression of cell cycle–related proteins in APN-KD myoblasts. The cyclin-dependent kinases CDKs form complexes with D-type cyclins to drive cell proliferation. A well-known target of the cyclin D–CDK4/6 complex is retinoblastoma protein (Rb), which inhibits cell cycle progression until it is inactivated by phosphorylation [19]. The levels of these proteins were significantly increased in APN-KD myoblasts (Fig. 3C). However, interestingly, the phosphorylation level of Rb significantly decreased in APN-KD myoblasts (Fig. 3C). Further, p21 inhibits the activity of cyclin D–CDK4/6 complexes, and thus functions as a regulator of cell cycle progression at G1 and S phase [20]. In the present study, the levels of p21 expression did not change in APN-KD myoblasts (Fig. 3C). Moreover, the expression levels of cleaved caspase-3 (C-Cas3), which plays a crucial role in apoptotic activity, was significantly increased in APN-KD myoblasts (Fig. 3C). To reveal the proliferative capacity of APN-KD myoblasts, we carried out the immunocytochemistry analysis of Ki67 which is a proliferation marker protein. As a result, the number of Ki67-positive cells was significantly decreased by APN suppression (Fig. 3D). Additionally, to investigate the cell damage of APN-KD myoblasts, we performed an assay to detect LDH (lactate dehydrogenase) activity in the supernatant, which is a stable enzyme that leaks from the cell upon cell plasma membrane damage. We found that LDH activity in the supernatant significantly increased in APN-KD myoblasts, which indicates that APN-KD led to apoptosis (Fig. 3E).

### 3.4. The formation of myotubes is suppressed in APN-knockdown myoblasts under the myogenic differentiation condition

To reveal the role of APN in myogenic differentiation, myogenic differentiation was induced in APN-KD myoblasts and the

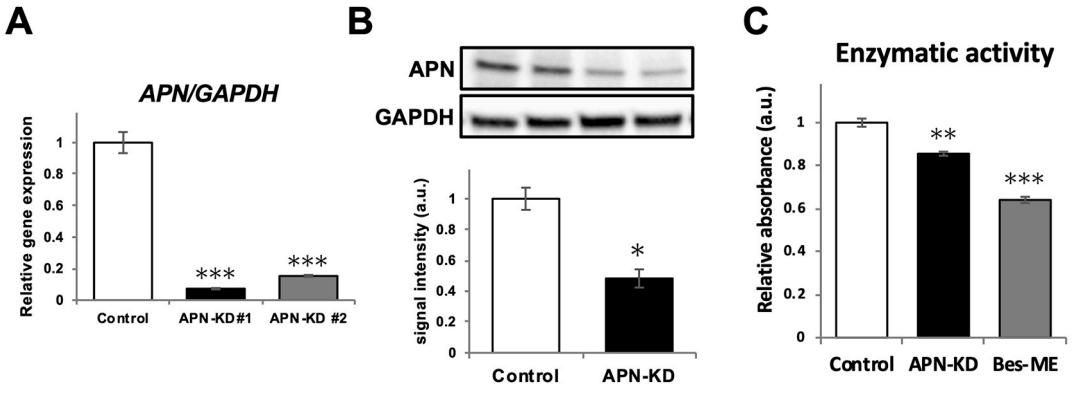


**Fig. 2.** Loss of alanine aminopeptidase (APN) expression suppresses enzymatic activity. (A) Real-time PCR analysis of expression of APN mRNA level in APN-KD myoblasts. APN knockdown led to a significant reduction in the expression of APN mRNA level in C2C12 myoblasts. Values are mean ± SEM (Student's t-test: ***$P < 0.001$ vs. Control; n = 3 per group). (B) Immunoblotting analysis of protein levels of APN in APN-KD myoblasts. APN knockdown led to a significant reduction in the expression of the APN level in C2C12 myoblasts. Values are mean ± SEM (Student's t-test: *$P < 0.05$ vs. Control; n = 3 per group). (C) Measurement of the aminopeptidase enzymatic activity against alanine substrate in APN-KD myoblasts or Bestatin methyl ester (Bes-ME) treated myoblasts. Bes-ME treatment markedly inhibited aminopeptidase enzymatic activity on the alanine substrate. Also, APN knockdown resulted in significant inhibition of the aminopeptidase enzymatic activity on alanine substrate in C2C12 myoblasts. Values are mean ± SEM (Student's t-test: ***$P < 0.001$ vs. Control; n = 6 per group).

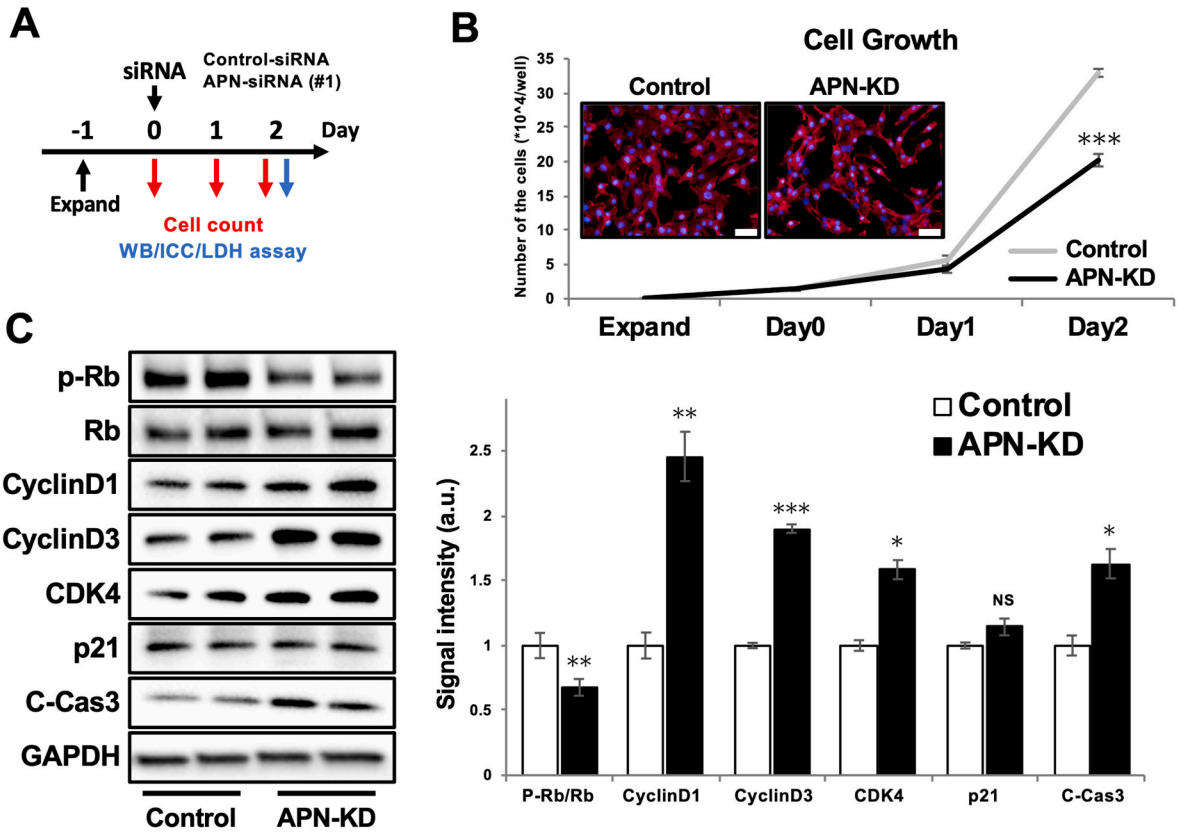


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**Fig. 3.** Loss of alanine aminopeptidase (APN) expression impairs cell cycle progression and induces apoptosis. (A) The time course of treatment and analysis in C2C12 myoblasts in which APN was suppressed using RNA interference (APN-KD). (B) Evaluation of cell growth 0, 1, 2 days after APN knockdown in C2C12 myoblasts, and staining of cell membrane using fluorescent phalloidin at 2 days after APN knockdown. Cell growth significantly decreased in APN-KD myoblasts at 2 days after knockdown treatment. Cell morphology changed in slender shape in APN-KD myoblasts. Scale bar: 50 μm. Values are mean ± SEM (Student's t-test: ***P < 0.001 vs. Control; n = 6 per group). (C) Immunoblotting analysis of protein levels of phosphorylation-Rb (p-Rb), Rb, Cyclin D1, Cyclin D3, CDK4, p21, cleaved-caspase-3 (C-Cas3), and GAPDH in APN-KD myoblasts at 2 days after knockdown treatment. The expression level of p-Rb was significantly decreased, whereas the expression of Cyclin D1, Cyclin D3, CDK4, C-Cas3 levels, except for p21, were significantly increased in APN-KD C2C12 myoblasts. Values are mean ± SEM. (Student's t-test: NS, not significant, **P < 0.01, ***P < 0.001 vs. Control; n = 3–6 per group). (D) Immunocytochemistry for Ki67 (red) and Hoechst 33342 (blue) in APN-KD myoblasts. The relative ratio of Ki67-positive cells was significantly reduced in APN-KD myoblasts. Scale bar: 100 μm. Values are mean ± SEM. (Student's t-test: **P < 0.01 vs. Control; n = 6 per group). (E) Evaluation of cell damage in APN-KD myoblasts at 2 days after knockdown treatment. LDH activity in the supernatant was significantly increased in APN-KD myoblasts. Values are mean ± SEM. (Student's t-test: NS, not significant, ***P < 0.001 vs. Control; n = 6 per group).

differentiation ability of the myoblasts was evaluated by immunocytochemical analysis of myosin heavy chain (MyHC). Myogenic differentiation was induced 2 d after APN-KD and morphological evaluation was performed 1, 3, 5, and 7 d thereafter (Fig. 4A). Myogenic differentiation delayed in APN-KD myoblasts (Fig. 4B). Consequently, the differentiation index, calculated as the ratio of the number of nuclei in myotubes with two or more nuclei to the total number of nuclei, was significantly decreased in APN-KD myotubes (Fig. 4C). Additionally, we revealed that APN-KD myotubes were significantly shorter than control myotubes (Fig. 4D).

### *3.5. Expression of myogenic differentiation factors is delayed in APN-knockdown myoblasts under the myogenic differentiation condition*

We investigated alterations in the expression of myogenic differentiation factors in APN-KD myoblasts during myogenic differentiation. Myogenic differentiation was induced 2 d after APN-KD, and the expression levels of MyoD, myogenin, and MyHC were evaluated 1, 3, 5, and 7 d thereafter (Fig. 5A). After 1 d of differentiation, the expression levels of these proteins were significantly decreased in APN-KD myoblasts (Fig. 5B). After 3 d of differentiation, the expression levels of MyHC and myogenin were significantly decreased, but the expression levels of MyoD did not differ from that in control myotubes (Fig. 5C). After 5 d of differentiation, the expression levels of MyHC was significantly decreased in APN-KD myoblasts, but the expression levels of myogenin and MyoD did not differ from those in control myotubes (Fig. 5D). After 7 d of differentiation, the expression levels of MyHC, myogenin, and MyoD did not differ from those in control myotubes (Fig. 5E).

## 4. Discussion

Since the proliferative capacity of myoblasts is essential for skeletal muscle formation, we examined the impact of APN gene suppression on proliferation in myoblasts. Our findings indicate that suppression of APN gene expression leads to a reduction in enzymatic activity, impairment of cell cycle progression, and apoptosis. Previously, administration of ubenimex, an inhibitor of APN/CD13, which is known as a stem cell marker protein, was reported to suppress cell proliferation and enhance apoptosis in cancer cells [21]. Also, blocking CD13/APN with an antibody was reported to induce apoptosis in melanoma cells [22]. These reports suggested that CD13/APN regulates cell proliferation and apoptosis in cancer cells, although the role of APN in myocytes has not previously been determined. In our recent study, we reported that treatment with Bes-ME suppresses enzymatic activity against alanine substrates and inhibits cell proliferation in C2C12 myoblasts, but does not induce apoptosis [11]. In contrast, in the present study, inhibition of cell proliferation and induction of apoptosis were observed in APN-KD myoblasts. Therefore, we concluded that the enzymatic activity of APN plays a role in cell proliferation, and that APN itself may function such as a signaling molecule in cell survival. Previous study have suggested that CD13/APN is a potent regulator of angiogenesis and its expression is induced by the mitogen-activated protein kinase (MAPK) pathway in endothelial cells [23]. Also, MAPK is indispensable for myogenesis-related processes, such as myoblast proliferation and differentiation has reported [24], suggesting APN may contribute to myogenesis through this pathway. However, it is unclear whether it participates in the signaling pathway through peptide degradation or by acting as a signaling molecule, so further studies are needed.

Rb is inactivated by cyclin–CDK complex phosphorylation, which drives the cell cycle [19,25]. In the present study, cyclin D1, cyclin D3, and CDK4 were upregulated, but the phosphorylation levels of Rb significantly decreased in APN-KD myoblasts. A previous study reported that overexpression of p16, a tumor suppressor gene, results in blocked cell division and, subsequently, a gradual reduction in Rb phosphorylation levels and the induction of apoptotic death, as in the present study [26,27]. Furthermore, MAPK pathways have been shown to control the expression levels of p16 in endothelial progenitor cells [28]. Also, MAPK pathways relate to CD13/APN has been reported [23]. We, therefore, assumed that APN may be involved in the regulation of Rb phosphorylation through the MAPK/p16 pathway.

Myogenic differentiation is a complex process controlled by the spatiotemporal expression of many myogenic regulatory factors and transcription factors and is indispensable for muscle maintenance and regeneration [29]. In the present study, delay in myogenic differentiation was observed in APN-KD myoblasts. The myogenic differentiation of myoblasts requires irreversible cell cycle withdrawal [30,31]. It has been shown that Rb protein is necessary for cell cycle progression and cell growth [32]. A previous study reported that inhibition of Rb interrupts the early stage of myogenic differentiation [33]. In this study, suppression of Rb phosphorylation was observed in APN-KD myoblasts. Therefore, we propose that impairment of myogenic differentiation in APN-KD myoblasts is caused by inhibition of Rb phosphorylation. Interestingly, a previous report suggested that Rb has the potential to be directly or indirectly involved in the function of MyoD [34,35]. Additionally, forced expression of cyclin D1 has been reported to inhibit the activity of MyoD [36]. In the present study, the expression levels of MyoD were significantly reduced after 1 day of myogenic differentiation, and the expression of cyclin D1 was upregulated in APN-KD myoblasts. Thus, we assumed that impairment of myogenic differentiation in APN-KD myoblasts might be caused by the reduction in MyoD expression that accompanies the repression of Rb phosphorylation and the upregulation of cyclin D1 expression.

It is widely known that peptide digestion by peptidases contributes to many cellular functions, including protein metabolism, intracellular signaling, and the production of peptide hormones [37]. To the best of our knowledge, this is the first report to indicate that APN is involved in cell cycle progression and myogenic differentiation in myoblasts. Since the role of intracellular aminopeptidases such as APN in myogenesis is still incompletely understood, further studies are required.

## 5. Conclusions

Knockdown of APN in C2C12 myoblasts resulted in impaired cell cycle progression and apoptosis. Furthermore, delayed myogenic differentiation was observed in APN-KD myoblasts. These findings suggest that APN contributes to the proliferation and differentiation of myoblasts.

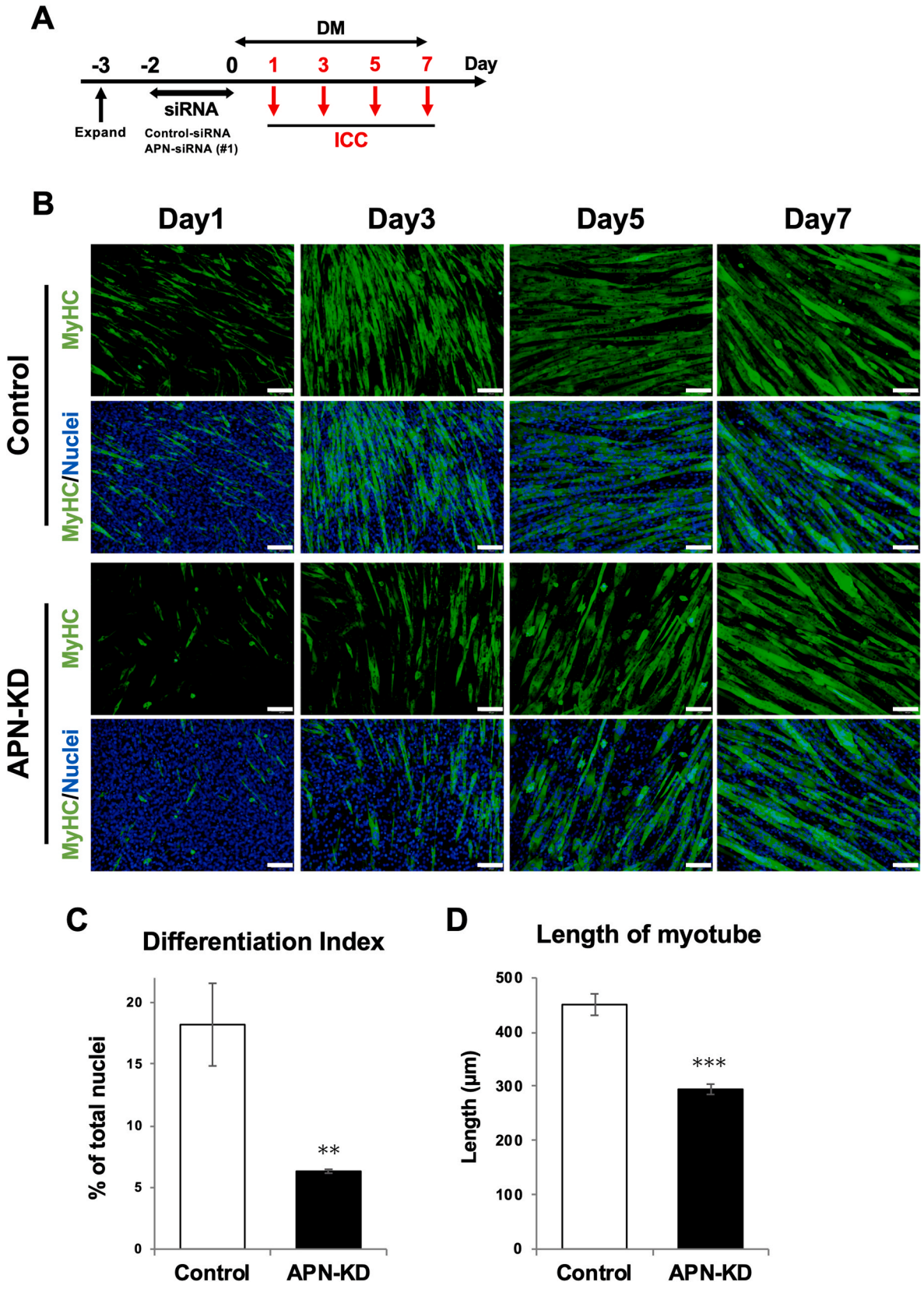


**Fig. 4.** The formation of myotubes is restricted in alanine aminopeptidase knockdown (APN-KD) C2C12 myoblasts under the myogenic differentiation condition. (A) The time course of treatment and analysis in APN-KD myotubes. (B) Immunocytochemistry for myosin heavy chain (MyHC; green) and nuclei (blue) in APN-KD myotubes after 1, 3, 5, and 7 days of myogenic differentiation. APN knockdown led to the delay in myogenic differentiation. Scale bar: 100 μm. (C) The differentiation index was significantly reduced in APN-KD myotubes after 3 days of myogenic differentiation. Values are mean ± SEM. (Student's t-test: ***P < 0.001 vs. Control; n = 5 per group, n = 100 number of myotube in each sample). (D) The length of myotubes was significantly shorter in APN-KD myotubes. Values are mean ± SEM. (Student's t-test: ***P < 0.001 vs. Control; n = 5 per group, n = 100 number of myotube in each sample).

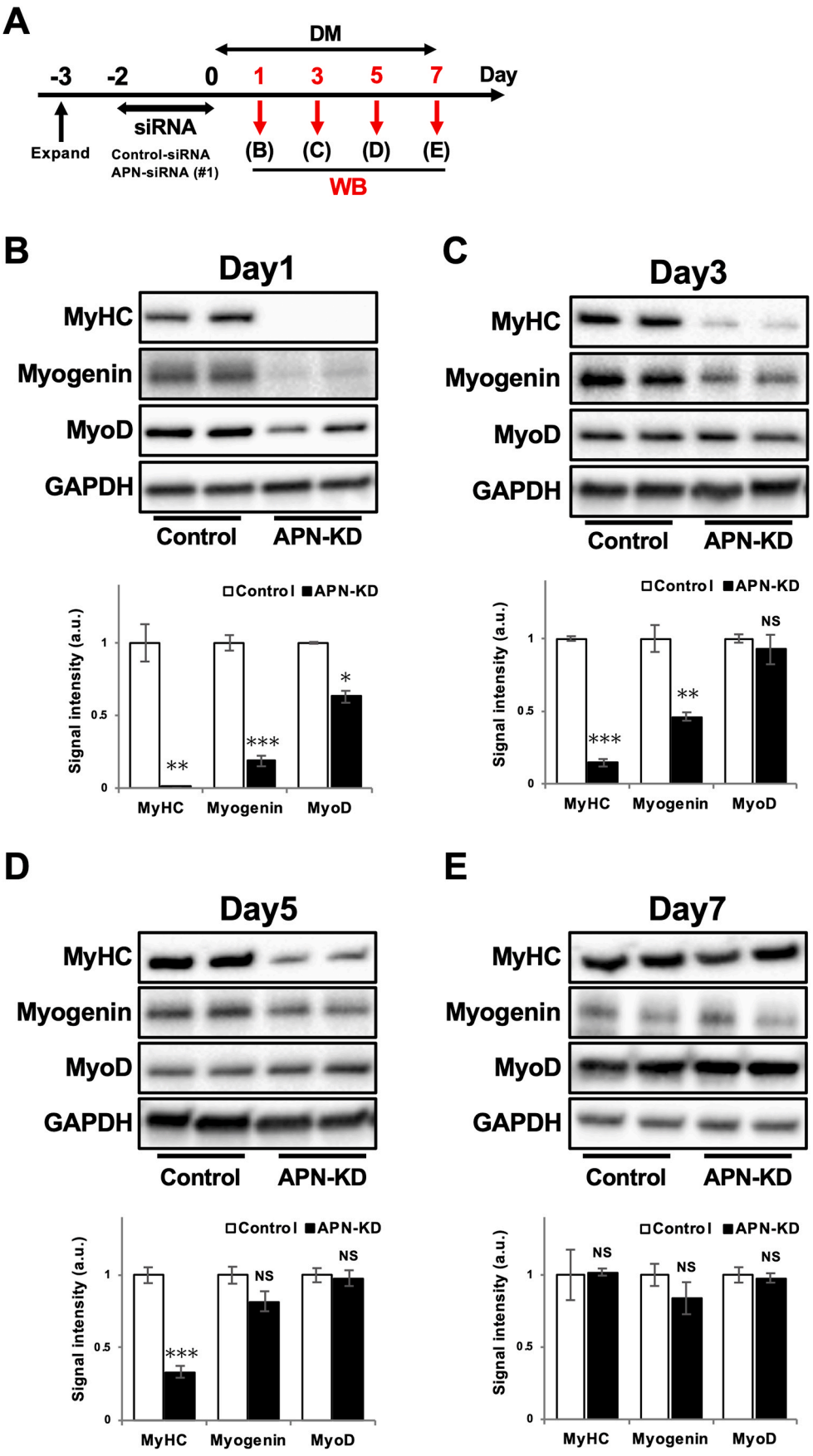


**Fig. 5.** Expression of myogenic differentiation factors is delayed in alanine aminopeptidase knockdown (APN-KD) C2C12 myoblasts under the myogenic differentiation condition. (A) The time course of treatment and analysis in APN-KD myotubes. Immunoblotting analysis of protein levels of myosin heavy chain (MyHC), Myogenin, MyoD, APN, and GAPDH after 1, 3, 5, and 7 days of myogenic differentiation in APN-KD myoblasts. (B) After 1 day of myogenic differentiation, MyHC, Myogenin, and MyoD expression levels were significantly decreased in APN-KD myoblasts. Values are mean ± SEM (Student's t-test: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control; n = 3 per group). (C) After 3 days of myogenic differentiation, MyHC and Myogenin except for MyoD expression levels were significantly decreased in APN-KD myoblasts. Values are mean ± SEM (Student's t-test: NS, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control; n = 3 per group). (D) After 5 days of myogenic differentiation, MyHC except for Myogenin and MyoD expression levels was significantly decreased in APN-KD myoblasts. Values are mean ± SEM (Student's t-test: NS, not significant, $^{***}P < 0.001$ vs. Control; n = 3 per group). (E) After 7 days of myogenic differentiation, MyHC, Myogenin, and MyoD expression levels did not change in APN-KD myoblasts. Values are mean ± SEM (Student's t-test: NS, not significant, $^{***}P < 0.001$ vs. Control; n = 3 per group).

## CRediT authorship contribution statement

**Shion Osana:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Project administration, Funding acquisition. **Yasuo Kitajima:** Validation, Formal analysis, Writing - review & editing. **Naoki Suzuki:** Validation, Formal analysis, Writing - review & editing. **Yidan Xu:** Validation, Formal analysis. **Kazutaka Murayama:** Validation, Formal analysis, Writing - review & editing. **Ryoichi Nagatomi:** Writing - review & editing, Conceptualization, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

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