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Original Article

PDZRN3 regulates adipogenesis of mesenchymal progenitors in muscle

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ABSTRACT

Introduction: Intramuscular adipose tissue (IMAT) is frequently formed in certain pathological conditions, such as biological aging, and ectopic fat accumulation leads to muscle weakness and a subsequent decline in physical function. Although mesenchymal progenitors (MPs) are present in postnatal skeletal muscle and are the cells from which IMAT originates, the molecular mechanism by which MPs contribute to IMAT formation has not been completely elucidated. Recently, we found that PDZ domain-containing ring finger 3 (PDZRN3), an E3-ubiquitin ligase, was highly expressed in MPs. In this study, we aimed to clarify the functions of PDZRN3 in MPs and the roles of PDZRN3 in IMAT formation using *in vitro* and *in vivo* experiments.

Methods: Primary mouse MPs isolated from hindlimb muscles were applied to adipogenic differentiation conditions, and expression fluctuation of PDZRN3 was verified with adipogenic differentiation and Wnt signaling markers. The role of PDZRN3 on MP's adipogenesis was evaluated *in vitro* by gene knock-down experiments. To evaluate the contribution of PDZRN3 to IMAT formation *in vivo*, tamoxifen-inducible MP-specific *Pdzrn3* knockout (*Pdzrn3*^{MPcKO}) mice were developed.

Results: PDZRN3 was more expressed in MPs than in muscle stem cells, and its expression profile of PDZRN3 fluctuated with the adipogenic differentiation of MPs. Our results revealed that PDZRN3 suppressed the adipogenesis of MPs *in vitro* through the activation of Wnt signaling and that a decrease in PDZRN3 accelerated adipogenesis. Indeed, IMAT significantly increased in the denervated muscles of *Pdzrn3*^{MPcKO} mice.

Conclusions: Our findings suggest that PDZRN3 is a key molecule in regulating IMAT formation. Since ectopic fat accumulation is frequently found in the skeletal muscles of older adults and also muscular dystrophy patients, PDZRN3 and its related pathways may represent a novel therapeutic target for these muscle pathologies.

1. Introduction

Sarcopenia is characterized by age-related attenuation of skeletal muscles and is clinically diagnosed by symptoms of muscle mass loss combined with muscle weakness or impaired physical function, not only by muscle mass loss [1]. Muscle weakness precedes the loss of muscle mass during aging and has a greater impact on physical function in older adults [2]. Muscle weakness in older adults may result from age-related structural changes in skeletal muscles, such

Abbreviations: IMAT, intramuscular adipose tissue; MP, mesenchymal progenitor; MuSC, muscle stem cell; PDZRN3, PDZ domain-containing ring finger 3; FAP, fibro/adipogenic progenitor; FBS, fetal bovine serum; DMEM, Dulbecco's modified eagle medium; ADM, adipogenic differentiation medium; IBMX, isobutyl-3-methylxanthine; ORO, oil-red O; PFA, paraformaldehyde.

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as fibrosis and fat deposition [3]. Fatty infiltration of skeletal muscles, known as intramuscular adipose tissue (IMAT), frequently occurs in older adults, leading to muscle weakness and a subsequent decline in physical function [4,5]. Since IMAT formation influences muscle quality and impairs the contractile function of muscles [6], IMAT is certainly one of the causes of sarcopenia. In other words, the inhibition of IMAT formation may be a novel therapeutic strategy for sarcopenia. However, the molecular mechanisms underlying IMAT formation have not been completely elucidated.

Mesenchymal progenitor (MP), also known as fibro/adipogenic progenitor (FAP), is a non-myogenic cell located in the interstitial space between muscle fibers and can be identified by the expression of several cell surface markers such as PDGFRα [7,8]. Acting as supporting cells that maintain the homeostasis of postnatal skeletal muscle, MPs can give rise to multiple cell lineages, including adipocytes, in response to some pathological conditions, indicating that they are the major cells of origin of IMAT [7,8]. Although it is unclear how fate decisions are regulated under pathological conditions, several molecular pathways are associated with lineage commitment to the adipocytes of MPs. For example, the canonical Wnt signaling inhibits MP adipogenesis, where Wnt5a binds to Frizzled to stimulate GSK3β, stabilizing β-Catenin in MPs [9]. However, the Wnt-associated regulation of MP adipogenesis has not been completely elucidated, particularly regarding intracellular signaling.

PDZRN3, a member of the PDZ domain-containing RING finger family, is highly expressed in skeletal muscle and regulates the terminal differentiation of myoblasts into myotubes through transcriptional and post-translational control of bHLH proteins [10,11]. In addition to myogenic differentiation, PDZRN3 is involved in many biological processes in the body. For example, PDZRN3 regulates vascular permeability by controlling endothelial junctional integrity [12] and negatively regulates adipogenic differentiation of 3T3L1 preadipocytes [13]. Although the molecular mechanisms underlying PDZRN3-mediated regulation of each biological process are not fully understood, Wnt signaling plays a key role in PDZRN3-dependent regulation. A recent proteomic analysis using mouse embryonic fibroblasts revealed that PDZRN3 is a downstream target of the Wnt-Ror signaling [14] and that Wnt5a stimulates the PDZRN3-dependent pathway in vascular endothelial cells [15].

In this study, we found that PDZRN3 was highly expressed in MPs of mouse skeletal muscles. Since the expression of PDZRN3 within postnatal skeletal muscle has been thought to be restricted in myogenic cells [11,16], our finding that it was highly expressed in MPs recalled a novel function for PDZRN3 in skeletal muscle. Therefore, we aimed to clarify the role of Pdzrn3, focusing mainly on MP adipogenesis and examined whether Pdzrn3 is involved in MP adipogenesis using primary cultured mouse MPs. We further developed MP-specific Pdzrn3 knockout mice and examined whether MP-specific Pdzrn3 deficiency affects IMAT formation *in vivo*.

2. Materials and methods

2.1. Cell isolation

Hind limb muscles were dissected from adult C57BL/6 N mice (Japan SLC). After carefully removing the tendons, nerves, blood vessels, and adipose tissues, the muscles were mechanically minced using scissors and enzymatically digested in 0.2 % collagenase type 2. After centrifugation, cell pellets were incubated with anti-α7-integrin (MBL), CD45 (Biolegend), and CD31 (Biolegend) antibodies and further incubated with anti-mouse IgG conjugated with microbeads (Miltenyi Biotech). The cell suspension was applied to an MS column (Miltenyi Biotech) to separate the positive and negative fractions of α7-integrin/CD45/CD31. The negative fraction was further incubated with an anti-Sca-1 antibody conjugated with microbeads (Miltenyi Biotech) and applied to an MS column to enrich the Sca-1$^+$/α7-integrin$^-$/CD45$^-$/CD31$^-$ fraction. The fraction of α7-integrin$^+$ fraction was used as muscle stem cells.

2.2. Cell culture, adipogenic differentiation, and siRNA treatment

Primary mouse MPs were plated on collagen-coated cell culture dishes and cultured in DMEM supplemented with 10 % FBS (growth medium). At the timing of appropriate confluency, the cell culture medium was replaced with the adipogenic differentiation medium (ADM) containing 1 μM of dexamethasone, 0.5 mM of isobutyl-3-methylxanthine (IBMX), and 10 μg/ml of insulin. Two days after lineage conversion, MPs were further cultivated in DMEM supplemented with 10 % FBS and 10 μg/ml insulin for 5–7 days.

To knock down *Pdzrn3*, the Accell siRNA delivery system was used according to the manufacturer's protocol (Horizon Discovery). Briefly, MPs were treated with 1 μM of siRNA (*Pdzrn3* or *Scramble*, Horizon discovery) diluted in the Accell siRNA delivery media for 48 h, and then the culture medium was replaced with ADM.

2.3. Immunocytochemistry

MPs were fixed with 4 % paraformaldehyde (PFA) for 15 min at room temperature for immunocytochemistry. The fixed cells were treated with 4 % normal goat serum/0.1 % Triton X/PBS and incubated with specific primary antibodies. Antibodies used in this study were anti-PDGFRα (Millipore), anti-TCF4 (Thermo Fisher Scientific), anti-PDZRN3 (Cell Signaling Technologies (CST)) [11], and goat anti-mouse/rabbit IgG conjugated with AlexaFluor 488 or 594 (Abcam). DAPI was used for nuclear staining.

2.4. Oil-Red O staining

Lipid droplets in adipogenic cells were visualized using Oil Red O (ORO, Fujifilm-Wako) on ADM days 5 or 7. The number of

Table 1
Primer sequences used for quantitative RT-PCR (qPCR).

Genes		Sequences (5′-3′)
Pdzrn3	Reverse	CTGACTCTTGTCCTGCATCGGGACTC
	Forward	ATGGGCTCCTTGGCTGTCTTGAAAGC
Cebpa	Reverse	CGACTTCAGCGCCTACATTGA
	Forward	CTAGCGACAGACCCCACAC
Pparg	Reverse	CCAGAGCATGGTGCCTTCGCT
	Forward	CAGCAACCATTGGGTCAGCTC
Sfrp1	Reverse	CAACGTGGGCTACAAGAAGAT
	Forward	GGCCAGTAGAAGCCGAAGAAC
Sfrp2	Reverse	CGTGGGCTCTTCCTCTTCG
	Forward	ATGTTCTGGTACTCGATGCCG
Sfrp4	Reverse	AGAAGGTCCATACAGTGGGAAG
	Forward	GTTACTGCGACTGGTGCGA
Sfrp5	Reverse	CACTGCCACAAGTTCCCCC
	Forward	TCTGTTCCATGAGGCCATCAG
Fzd1	Reverse	CAGCAGTACAACGGCGAAC
	Forward	GTCCTCCTGATTCGTGTGGC
Fzd2	Reverse	GCCGTCCTATCTCAGCTATAAGT
	Forward	TCTCCTCTTGCGAGAAGAACATA
Fzd4	Reverse	GCCCCACAAGACTCCCATC
	Forward	CCAGCATCGTAGCCACACT
Fzd7	Reverse	GCCACACGAACCAAGAGGAC
	Forward	CGGGTGCGTACATAGAGCATAA
Fzd9	Reverse	GCTGGAGAAGCTGATGGT
	Forward	CCAGAGAGGGGTCTGTCT
Plin1	Reverse	TGAAGCAGGGCCACTCTC
	Forward	GACACCACCTGCATGGCT
Plin2	Reverse	CCCGTATTTGAGATCCGTGT
	Forward	CAATTTGTGGCTCCAGCTTC
β-actin	Reverse	GCAGTGAAGAATGCACACGA
	Forward	CAAGCAAAGTCAGCACCACT

adipocytes was counted in five randomly chosen fields in each well, and the percentage of ORO^{+} cells in each field was quantified using Image J software (NIH).

2.5. Quantitative RT-PCR (qPCR)

Total RNA was extracted from primary mouse MPs using TRI Reagent (Molecular Research Center) at the appropriate time points, and cDNA was synthesized using the PrimScript II 1st strand cDNA synthesis kit (Takara Bio). Polymerase chain reaction (PCR) was performed using the CFX96 real-time PCR detection system (Bio-Rad) and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The PCR primers used in this study are listed in Table 1.

2.6. Western blotting

Proteins were extracted from primary mouse MPs using SDS-HBS (1 % SDS/150 mM NaCl/10 mM HEPES, pH 7.4). Twenty micrograms of each sample were used for polyacrylamide gel electrophoresis and then electroblotted using a PVDF membrane.

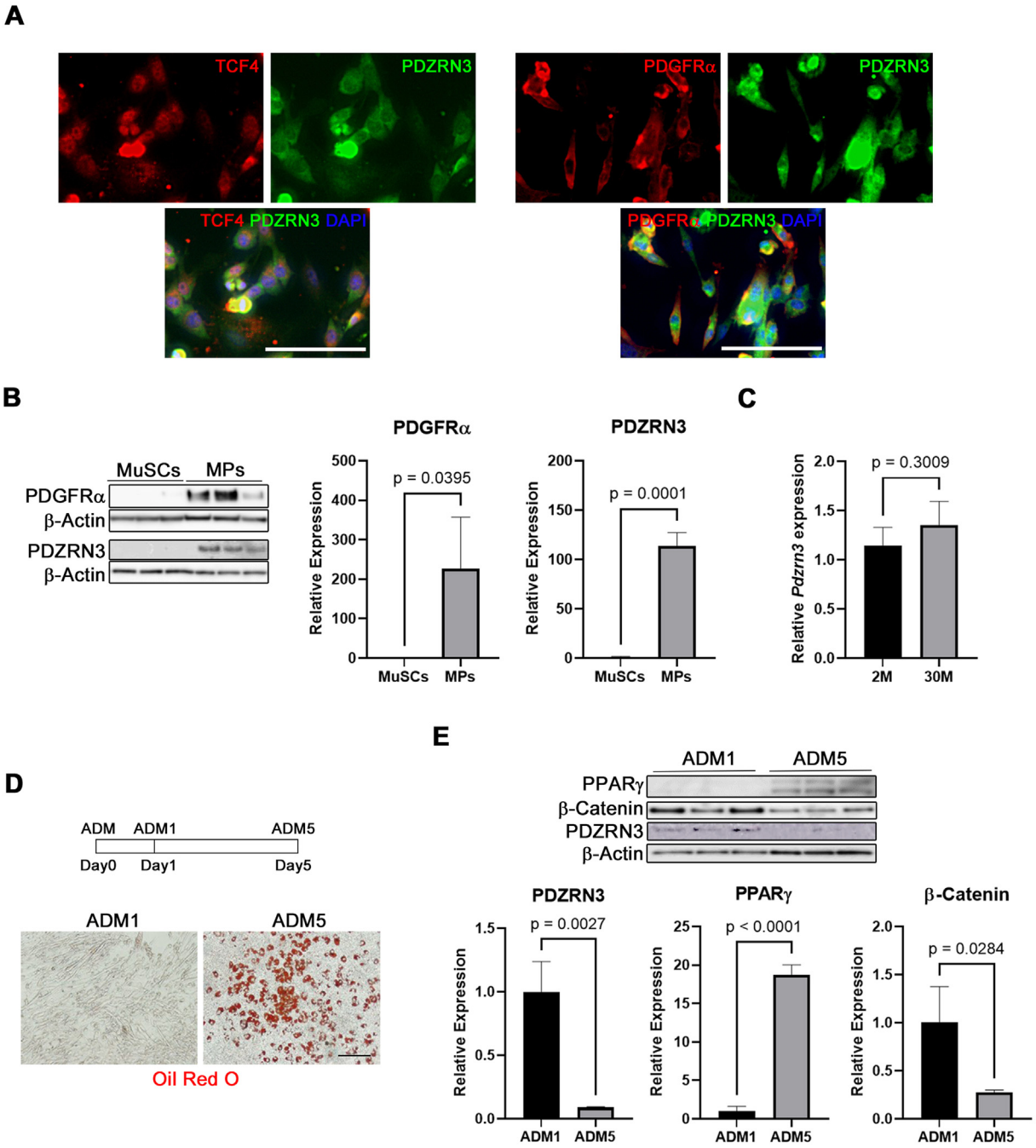

Fig. 1. PDZRN3 is highly expressed in mesenchymal progenitors. (A) MPs isolated from mouse hindlimb muscles co-expressed PDGFRα (Red) or TCF4 (Red) and PDZRN3 (Green). Scale bar = 100 μm. (B) Western blot analysis for PDGFRα and PDZRN3 in mouse muscle stem cells (MuSCs) and MPs. $n = 3$. (C) PDZRN3 gene expression was compared between young (2 months; 2 M) and aged (30 months; 30 M) mouse-derived MPs. $n = 3$. (D) Primary MPs were differentiated into adipocytes by cultivation in adipogenic differentiation medium (ADM) for 5–7 days. Oil-Red O staining was performed to visualize adipocytes at ADM1 (ADM Day 1) and ADM5 (ADM Day 5). Scale bar = 100 μm. (E) Western blot analysis for β-Catenin and PDZRN3 in MPs at ADM1 and AMD5. $n = 3$.

Following blocking with 5 % skim milk/PBS-Tween 20, the membrane was incubated with the primary antibody overnight at 4 °C. The following primary antibodies were used in this study: anti-PDZRN3 (Protein*Express*), anti-PPARγ (Santa Cruz Biotechnology), anti-PDGFRα, anti-β-Catenin, and anti-GSK3β (CST), sFRP4 (Proteintech), β-Actin (Proteintech), The secondary antibodies used in this study were HRP-conjugated anti-rabbit IgG and anti-mouse IgG (CST).

2.7. MP-specific Pdzrn3-knockout mouse

Pdgfra^{CreER} mice (Jackson Laboratory) were crossed with *Pdzrn3*^{flox} mice [16] to generate *Pdgfra*$^{CreER/wt}$:*Pdzrn3*$^{flox/flox}$ mice. To induce Cre-mediated recombination and MP-specific *Pdzrn3* deficiency, 12-week-old mice were intraperitoneally injected with 1 mg tamoxifen per 10 g body weight for 5 days [17]. *Pdzrn3*^{MPcko} and wild-type mice were used for denervation. To induce denervation, sciatic nerves in the right leg were resected under anesthesia with Medetomidine hydrochloride (0.3 mg/kg), Midazolam (4 mg/kg), and Buprenorphine (5 mg/kg), and the denervated mice were housed for 3 weeks. As a control, a sham operation was performed on the left legs of *Pdzrn3*^{MPcko} and wild-type mice. Three weeks after surgery, mice were sacrificed by cervical dislocation under anesthesia. *Tibialis Anterior* and *Gastrocnemius* muscles were collected from both the right and left legs for qPCR and histological analyses. For histological analysis, 7-μm thick frozen muscle cross sections (*Gastrocnemius*) were used for immunofluorescent staining of Perilipin (CST) and Laminin-α2 (Thermo Fisher Scientific). The IMAT was calculated using the perilipin^{+} area on the entire surface of the muscle cross-section.

All mice were maintained, crossed, genotyped, and sacrificed in accordance with the Institutional Animal Care and Use Committee of the National Center for Geriatrics and Gerontology (no. 5–7 and 5–9).

2.8. Statistical analysis

All statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software).

3. Results

3.1. PDZRN3 is highly expressed in mesenchymal progenitors in postnatal skeletal muscle

Mesenchymal progenitors (MPs) and muscle stem cells (MuSCs) were isolated from mouse hind limb muscles (2–6 months old) by magnetic bead separation. Isolated MPs co-expressed PDGFRα or TCF4, and PDZRN3, and the expression of PDZRN3 was higher in MPs than in MuSCs (Fig. 1A and B), indicating that MPs are one of the major cell types expressing PDZRN3 in postnatal skeletal muscle, in addition to myoblasts [11]. In addition, the expression of *Pdzrn3* was not altered in aged mouse-derived MPs (Fig. 1C), suggesting that age did not affect *Pdzrn3* expression.

Mouse MPs were cultured in adipogenic differentiation medium (ADM) to analyze changes in PDZRN3 expression during adipogenesis (Fig. 1D). Expression of PPARγ was increased on ADM day 5 (ADM5), which was consistent with Oil Red O staining (Fig. 1D and E). In contrast, PDZRN3 and β-Catenin expression levels significantly decreased in ADM5 cells (Fig. 1E). These results suggest a possible association between PDZRN3 expression and Wnt signaling in the progression of MP adipogenesis.

3.2. PDZRN3 regulates Wnt signaling-associated gene expression

To investigate the interaction between PDZRN3 and Wnt signaling, *Pdzrn3* was knocked down in primary cultured mouse MPs, and the expression of sFRP and Frizzled genes was evaluated by qPCR analysis. Forty-eight hours after si*Pdzrn3* administration,

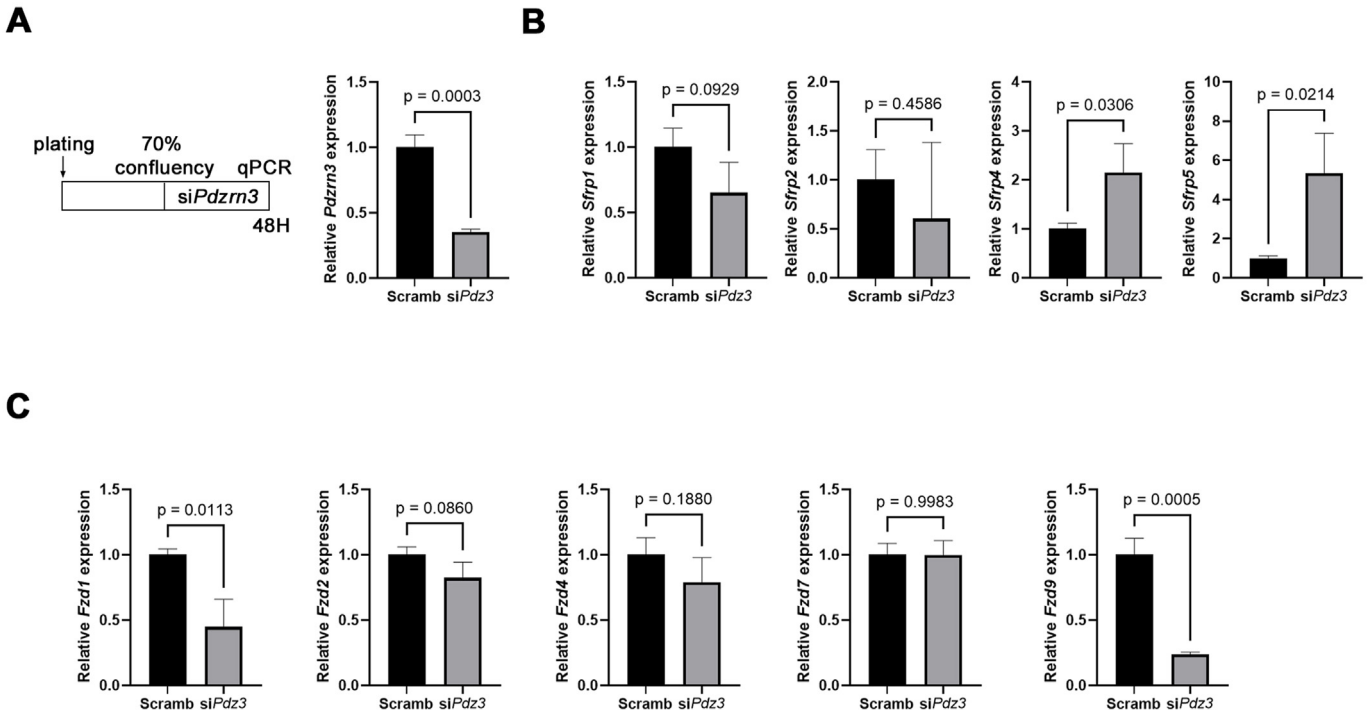

Fig. 2. *Pdzrn3* interference alters sFRP and Frizzled gene expression in MPs. (A) si*Pdzrn3* (si*Pdz3*) or si*Scramble* (Scramb) was added into the serum-free medium when cell density reached 70 % confluency, and then cells were cultivated for 48 h to interfere with *Pdzrn3*. *Pdzrn3* expression was analyzed by qPCR. $n = 3$. (B, C) Expression of *Sfrp* and *Fzd*, which were Wnt signaling-related genes, was analyzed in si*Pdzrn3*-treated mouse MPs. $n = 3$.

Pdzrn3 expression significantly decreased (Fig. 2A) and *Sfrp4* and *Sfrap5* expression significantly increased in si*Pdzrn3*-treated MPs (Fig. 2B). Since sFRPs are soluble Wnt inhibitors, PDZRN3 may contribute to the regulation of Wnt signaling. We also found that the expression of *Fzd1* and *Fzd9*, which are involved in Wnt signaling, significantly decreased in si*Pdzrn3*-treated MPs (Fig. 2C).

3.3. Downregulation of PDZRN3 accelerates MP adipogenesis by inhibiting Wnt signaling

To determine whether the downregulation of PDZRN3 expression influences MP adipogenesis, MPs were cultured for 48 h under adipogenic differentiation conditions following si*Pdzrn3* treatment (Fig. 3A). At day 7 of ADM, we confirmed that MPs differentiated into adipocytes owing to the upregulation of PPARγ expression. Inhibition of *Pdzrn3* by siRNA deregulated sFRP4 and- β-Catenin expression similar to non-adipogenic differentiation conditions. In addition to downregulating GSK3β expression, si*Pdzrn3* treatment might have directly deregulated the expression of Wnt signaling-associated factors (Fig. 3B and C). Since Wnt signaling is involved in the adipogenesis of various stem/progenitor cells [18], the altered expression of Wnt-associated factors following *Pdzrn3* knockdown might affect the adipogenic differentiation capacity of MPs. Indeed, si*Pdzrn3* treatment significantly increased the number of Oil Red O^{+} mature adipocytes (Fig. 3D).

3.4. Pdzrn3 deficiency accelerates IMAT formation in vivo

The results of the *in vitro* experiments using si*Pdzrn3* prompted us to investigate whether *Pdzrn3* ablation accelerates fatty infiltration in skeletal muscle. To induce *Pdzrn3* deficiency specifically in MPs, *Pdgfra*^{CreER} mice were crossed with *Pdzrn3*^{flox} mice, followed by intraperitoneal injection of tamoxifen to generate *Pdzrn3*^{MPcKO} mice (Fig. 4A). *Pdzrn3* expression was significantly decreased in the muscle tissue of *Pdzrn3*^{MPcKO} mice, indicating that *Pdzrn3* was successfully ablated in MPs by tamoxifen injection (Fig. 4B). The sciatic nerves in the right legs of *Pdzrn3*^{MPcKO} mice were resected to induce muscle atrophy, and the formation of IMAT and expression of adipogenesis-related genes (adipo-genes) were evaluated 3 weeks after denervation. Although denervation increased adipose-gene expression even in wild-type mice, its impact was strengthened by MP-specific *Pdzrn3* ablation, and the Perilipin^{+} area in the muscle cross-section, which was defined as IMAT, was significantly larger in *Pdzrn3*^{MPcKO} mice (Fig. 4C and D). Taken together with the results from the *in vitro* experiments, a decrease in PDZRN3 expression accelerated adipogenic differentiation of MPs and consequently increased IMAT formation in adult skeletal muscle.

4. Discussion

In the present study, we detected PDZRN3 in MPs higher than in activated muscle stem cells. Although PDZRN3 is

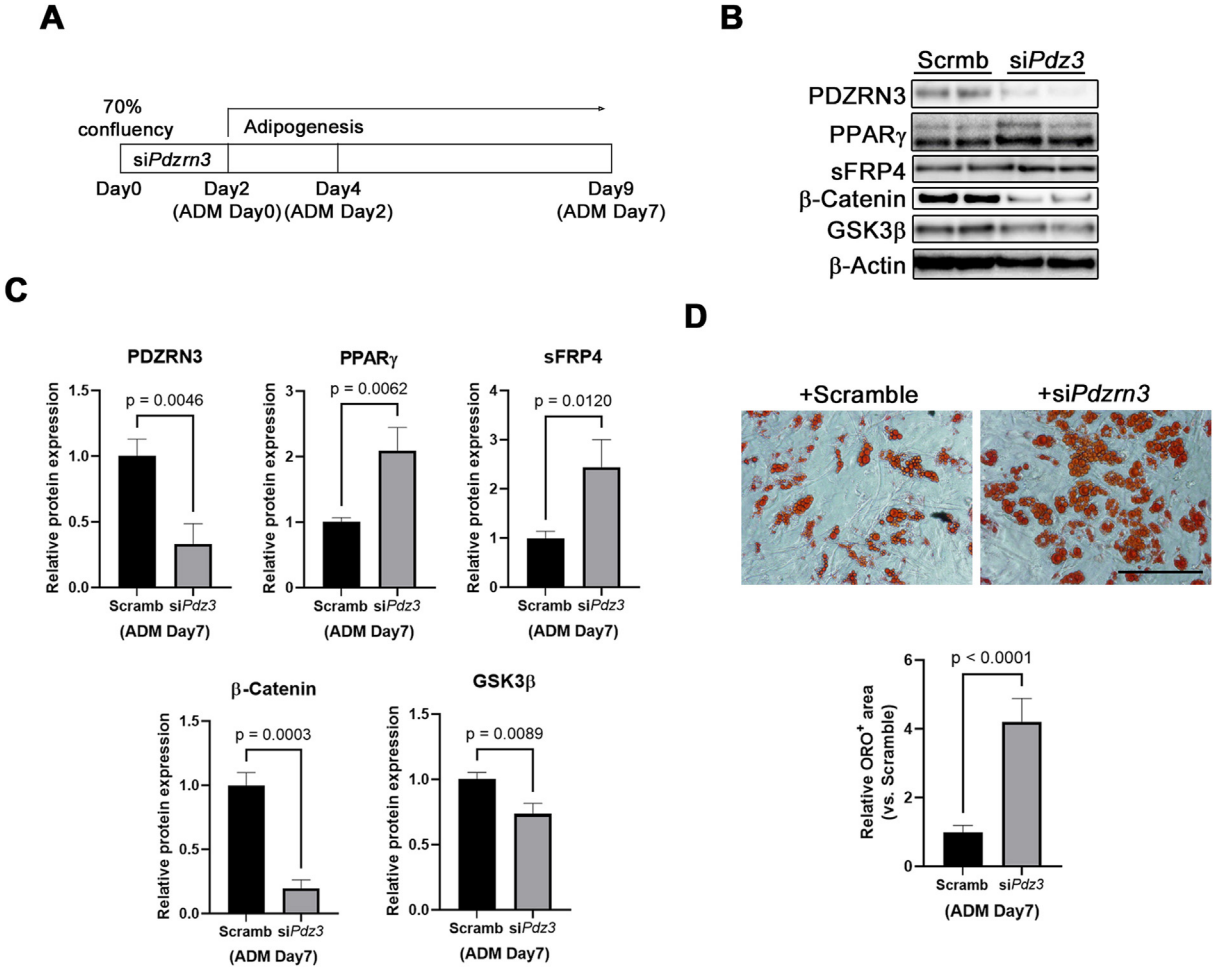

Fig. 3. Decreased *Pdzrn3* accelerates adipogenesis of MPs. (A) Adipogenic differentiation was applied following siRNA treatment, and then an extent of adipogenesis was evaluated by calculating marker expression at ADM Day 7. (B, C) Western blot analysis for PPARγ, sFRP4, β-Catenin, and GSK3β in si*Pdzrn3*-treated MPs at ADM Day7. $n = 3$. (D) Oil-Red O (ORO) staining was performed at ADM Day 7. Then, the ORO $^{+}$ area was calculated and compared between the Scramb and si*Pdz3*. $n = 3$. Scale bar = 100 μm.

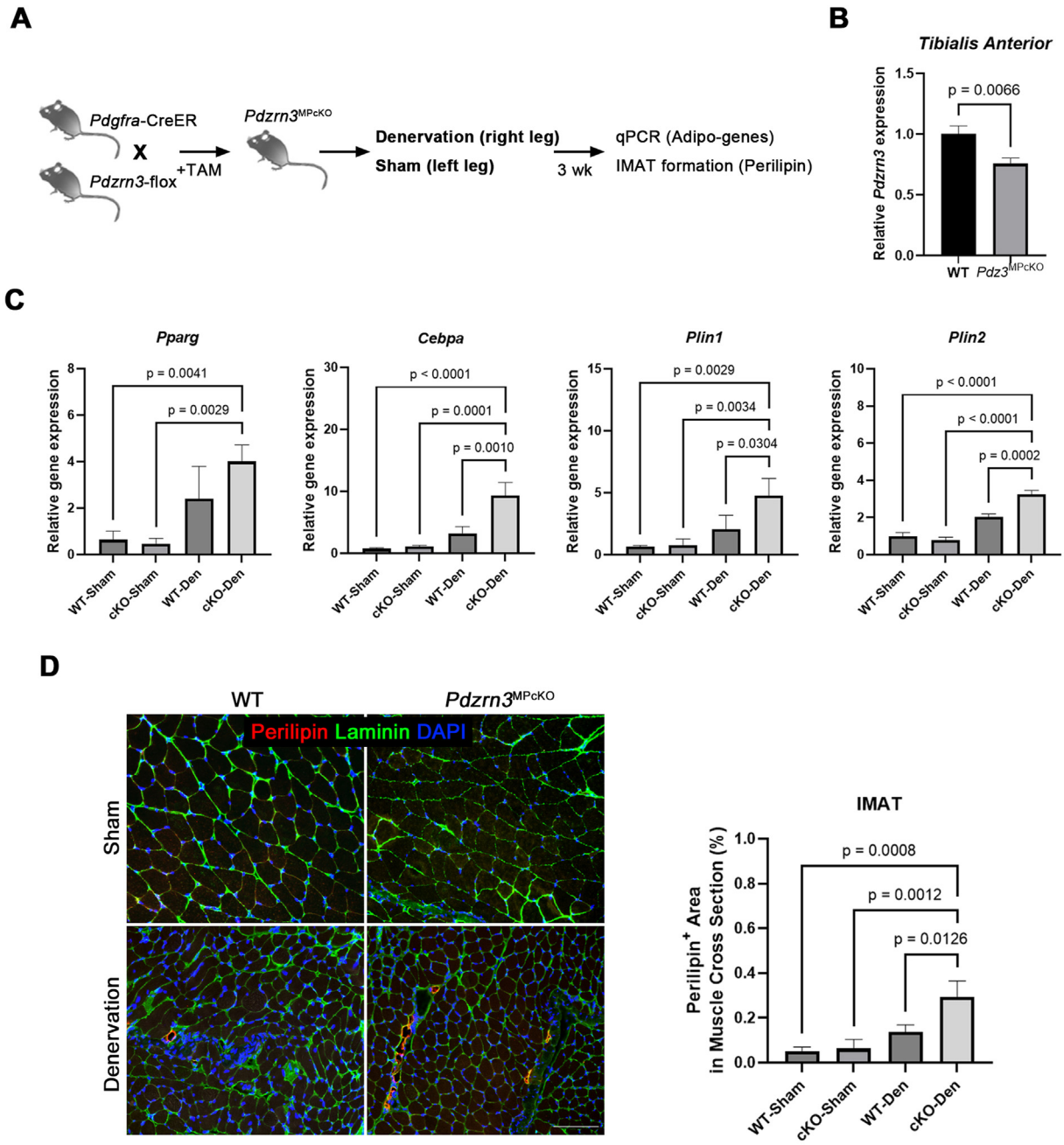

Fig. 4. *Pdzrn3* deficiency in MPs expands IMAT in denervated skeletal muscle. (A) Strategy for developing MP-specific *Pdzrn3*-conditional knockout ($Pdzrn3^{MPcKO}$) mice. $Pdgfra^{CreER}$ mouse was crossed to $Pdzrn3^{flox}$ mouse, and then tamoxifen (TAM) was intraperitoneally injected into mice. $Pdzrn3^{MPcKO}$ mice were subjected to surgical operations for denervation (right leg) or sham (left leg) and then sacrificed 3 weeks after the operation to evaluate adipo-gene expression and IMAT formation. (B) *Pdzrn3* expression was evaluated by qPCR in muscle tissue (*Tibialis Anterior*) of $Pdzrn3^{MPcKO}$ mice. $n = 3$. (C) The expression of adipo-genes in muscle tissue was analyzed using qPCR. $n = 3$. (D) An occupancy of fat (IMAT) area in muscle cross-section was analyzed by immunofluorescence for Perilipin. $n = 3$. WT-Sham; sham-operated wild-type mouse, cKO-Sham; sham-operated $Pdzrn3^{MPcKO}$ mice, WT-Den; denervation-induced wild-type mice, cKO-Den; $Pdzrn3^{MPcKO}$ mice. Scale bar = 100 μm.

predominantly expressed in skeletal muscles and is essential for the terminal differentiation of myoblasts [10,11], its high expression in intramuscular non-myogenic cells suggests an important role in MP. PDZRN3 affects cell differentiation and tissue development and mediates Wnt signaling during some developmental events. For example, PDZRN3 regulates vascular morphogenesis through Wnt/Planar cell polarity (PCP) signaling. In vascular development, PDZRN3 stabilizes both tight junctions and the polarity shift of endothelial cells [15]. Forced expression of *Pdzrn3* in vascular endothelial cells causes abnormal junctions of endothelial cells through Wnt signaling, accompanied by severe bleeding and cognitive impairment [12,19]. Forced expression of PDZRN3 in cardiomyocytes causes abnormal heart development with alteration of ZO-1 and Connexin-43 expression [20], and junctional and polarization control of cells might be one of the targets of PDZRN3 in cardiovascular cells. Moreover, PDZRN3 also regulates the progression of endometrial carcinoma via the canonical Wnt pathway [21]. In the case of MPs, the expression of endogenous PDZRN3 decreased with adipogenesis progression, and ablation of this gene enhanced adipogenic differentiation

capacity, suggesting that PDZRN3 acts as a suppressor of adipogenesis in MPs.

Although our understanding of the PDZRN3-associated molecular pathway is poor, previous studies have supported a relationship between PDZRN3 and Wnt signaling. Since Wnt signaling is a principal factor in the adipogenic differentiation of several types of stem/progenitor cells [18,22,23], it is reasonable to consider the role of PDZRN3 in MP adipogenesis. In the present study, we found that sFRP4, which antagonizes Wnt signaling by binding to either the Wnt ligand or Frizzled [24], was upregulated by si*Pdzrn3* treatment under both non-adipogenic and adipogenic culture conditions. The fact that sFRP4 expression increased in response to si*Pdzrn3* without adipogenic stimulation indicated a direct relationship between PDZRN3 and sFRP4. Although further investigation is needed to prove whether the upregulation of sFRP4 directly affects adipogenesis in MPs, a previous study showing that *Sfrap4* interference suppresses adipogenesis of human adipose tissue-derived mesenchymal stem cells might support the possibility of a direct interaction between PDZRN3 and sFRP4 [25]. On the other hand, it has also been reported that PDZRN3 regulated adipogenesis in 3T3-L1 cells through the STAT5b-C/EBPβ pathway [13], suggesting that there are multiple pathways in PDZRN3-mediated inhibition of adipogenesis. It is currently unknown whether STAT5 signaling is involved in PDZRN3-mediated suppression of adipogenesis in MP as well as 3T3L1 cells, but there may be differences in the PDZRN3-mediated adipogenesis pathway among different cell types.

During adipogenesis, PDZRN3 expression in MPs gradually decreases as differentiation progresses, indicating that PDZRN3 transcription is strictly controlled during differentiation. However, the mechanism underlying the transcriptional regulation of PDZRN3 in all cell types is poorly understood. Positive Regulatory Domain Zinc Finger Region Protein 16 (PRDM16) is the only factor that directly regulates PDZRN3 expression by silencing *Pdzrn3* to control the migration of cortical neurons [26]. Since PRDM16 acts in multiple steps during differentiation in preadipocytes [27] and is highly expressed in MPs [28], it is expected that PRDM16 controls PDZRN3 transcription during MP adipogenesis.

In this study, we also found that adipogenesis was accelerated in MPs by downregulating *Pdzrn3* expression, indicating that lower levels of PDZRN3 might result in the formation of adipocytes such as IMAT. This hypothesis was supported by the enhanced fatty infiltration of muscles in denervation-induced $Pdzrn3^{MPcKO}$ mice, although denervation-induced IMAT formation was observed even in wild-type mice. As there was no contradiction in PDZRN3 expression between young and aged mouse-derived MPs, environmental changes surrounding the MPs may influence PDZRN3 expression. Another interesting point was the lack of IMAT formation in sham-operated skeletal muscle despite the MP-specific PDZRN3 deficiency. Since similar results have been observed in a previous study using MP-specific vitamin D receptor-deficient mice [29], these results suggest that IMAT formation requires not only changes within the MP but also other changes within skeletal muscle. Additional factors within skeletal muscle required for IMAT formation have not yet been identified. Still, it is possible that degenerated myofibers, which are frequently observed in pathological skeletal muscles, are another essential factor required for IMAT formation *in vivo* [30]. Further detailed investigations are needed to clarify the mechanism of IMAT formation in a future study. Ectopic fat accumulation in muscles reduces muscle quality, leading to muscle weakness and sarcopenia. Therefore, more attention should be paid to age-related changes in muscle quality, although research on sarcopenia has focused on muscle mass [31].

5. Conclusions

In this study, we demonstrated, using both *in vitro* and *in vivo* models, that PDZRN3 suppresses MP adipogenesis in skeletal muscle. This effect was mediated, at least in part, by the regulation of Wnt signaling-related factors such as sFRP4. Although further detailed investigations are needed, our findings suggest that PDZRN3 and its related pathways might be key molecular players in IMAT formation in some pathological conditions. Since ectopic fat accumulation is frequently found in the skeletal muscles of older adults and also muscular dystrophy patients, PDZRN3 and its related pathways may represent a novel therapeutic target for these muscle pathologies.

Ethics approval

The study was conducted in accordance with the relevant guidelines and carried out in compliance with the ARRIVE guidelines. The methods were carried out in accordance with the approved guidelines and the ARRIVE guidelines.

Data availability

Data will be made available on request.

Authorship contribution statement

H.I., Y.T., S.I., and T.H. conceived and designed the study. H.I., M.K-T., Y.M., A.U., T.H., and T.H. acquired the data. H.I., M.K-T., Y.M., and T.H. analyzed and interpreted the data. H. I. and T.H. wrote the manuscript. All the authors have read and approved the final version of this manuscript.

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Declaration of competing interest

The authors have no conflicts of interest to disclose.

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