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Actin-associated protein palladin is required for migration behavior and differentiation potential of C2C12 myoblast cells

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ABSTRACT

The actin-associated protein palladin has been shown to be involved in differentiation processes in non-muscle tissues. However, but its function in skeletal muscle has rarely been studied. Palladin plays important roles in the regulation of diverse actin-related signaling in a number of cell types. Since intact actin-cytoskeletal remodeling is necessary for myogenesis, in the present study, we pursue to investigate the role of actin-associated palladin in skeletal muscle differentiation. Palladin in C2C12 myoblasts is knocked-down using specific small interfering RNA (siRNA). The results show that down-regulation of palladin decreased migratory activity of mouse skeletal muscle C2C12 myoblasts. Furthermore, the depletion of palladin enhances C2C12 vitality and proliferation. Of note, the loss of palladin promotes C2C12 to express the myosin heavy chain, suggesting that palladin has a role in the modulation of C2C12 differentiation. It is thus proposed that palladin is required for normal C2C12 myogenesis *in vitro*.

1. Introduction

Skeletal muscle is a heterogeneous tissue that is required for almost all vertebrate conscious action. The differentiation of muscle progenitor cells into mature myotubes, called myogenesis, is a highly ordered process [1–3]. Initially, mononucleoted myoblasts proliferate, irreversibly withdraw from the cell-cycle, migrate and align with one another. After alignment, myoblasts fuse to form multinucleated myotubes and terminal differentiated myofibers [4,5]. Skeletal muscle differentiation necessitates intensive actin cytoskeleton rearrangement to undergo differentiation with complex changes, including maintenance of cell shape, coordinated cell locomotion, adhesion, fusion, cell–extracellular matrix interaction, and muscle contraction [6]. Growing evidence suggests that the sub-cellular coordination of the cytoskeleton and its regulatory, scaffolding and cytoskeletal cross-linking proteins are responsible for these dynamic reorganizations and maintaining the normal actin-cytoskeleton during myogenesis [7–9].

Palladin was characterized independently by two research groups, namely those of Dr. Otey and Dr. Carpén, a decade ago [10,11]. This phosphoprotein belongs to the cytoskeleton-associated protein family named palladin–myotilin–myopalladin [12]. Palladin functions as a scaffold protein that interacts with actin and numerous actin-associated proteins that are required for organizing the actin-cytoskeleton [13–16]. Palladin is widely expressed in both muscle and non-muscle cells and tissues. In vertebrates, several palladin isoforms are transcribed from a single gene through alternative splicing [12,17,18]. Three canonical isoforms of palladin have been exhaustively characterized, with molecular weights of 200, 140, and 90 kDa, respectively [10,19]. Over the past few years, many studies have suggested the contribution of palladin in organizing actin arrays, which is necessary for controlling cell shape, migration, invasion, and development in a variety of cell types and tissues [10,16,19,20]. Indeed, palladin presents in actin-based structures such as stress fibers, focal adhesions, membrane ruffles, and podosomes [10,21]. Palladin knockout mouse had the embryonic lethal phenotype due to neural tube closure defects [18]. In addition, suppression of palladin expression in neurons resulted in decreasing of neurite outgrowth [22]. Moreover, fibroblasts derived from palladin-deficient mice showed defects in cell motility, adhesion, and actin organization [23]. Nevertheless, the overexpression of palladin in Cos-7 cells and astrocytes increased the number and size of actin bundles [24]. Importantly, 140 kDa-palladin was up-regulated by TGF-β1 during myofibroblast differentiation [25]. Likewise, palladin also played an important role in smooth muscle cell differentiation [26]. However, fewer studies have reported the role of palladin in skeletal muscle differentiation.

Given that palladin is involved in the regulation of diverse actin-related signaling processes, it is speculated that palladin plays potential roles in skeletal muscle differentiation, at least through

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actin-cytoskeleton remodeling activities. The present study examines whether palladin is related to the differentiation of murine skeletal muscle C2C12 myoblasts. Our data demonstrat for the first time that a depletion of palladin by siRNA inhibit the migration of skeletal muscle cells, but promot their proliferation and differentiation.

2. Materials and methods

2.1. Mouse skeletal muscle cell line C2C12 culture

C2C12 (from Bioresource Collection and Research Center, Taiwan) was propagated as myoblasts in growth medium, GM (Dulbecco's modified Eagle's medium, DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere of air and 5% CO_2. For differentiation into myotubes, the myoblasts were switched to differentiation medium, DM (DMEM with 2% horse serum (GIBCO, USA)). The myotubes began to form in 2–4 days post-differentiation.

For 3-(4, 5-dimethyl thiazol–2-yl-)-2, 5-diphenyl tetrazolium bromide (MTT) and 5-bromo-2-deoxyuridine (BrdU) assays, myoblasts were grown in GM at initial densities of 1000 cells/well on 96-well culture plates. After 1 day, cells were transfected with siRNA for 24 h, then shifted to DM for 0–72 h.

2.2. RNA oligonucleotides and transfection

All RNA oligonucleotides used in this study were synthesized from MDBio (Taiwan). Four sequences of siRNA (siRNA1–siRNA4), each complementary to a unique segment of mRNA transcribed from the coding sequences common to all isoforms of the murine palladin gene, were designed. One scrambled siRNA was used as a control (Ctrl). Target sequences were aligned to the mouse genome database in a BLAST search to eliminate sequences with significant homology to other genes. The oligonucleotides sequences are listed in Supplementary Table 1. C2C12 cells were aliquoted into plates 24 h before transfection. Cells were transfected with palladin siRNA or control non-targeted siRNA using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions (day 0). Differentiation was induced 24 h post-transfection and indicated as day 1. For all experiments, transfection was conducted when the cells were at approximately 50% confluence.

2.3. Measurement of cell vitality (MTT assay)

Cell viability was assessed *via* MTT assay (Sigma–Aldrich). At the indicated time points, the medium was aspirated and cells were incubated with fresh medium containing 0.2 mg/mL MTT for an additional 4 h at 37 °C. After incubation, the medium was removed and the formazan crystals were dissolved in 100 mL dimethyl sulfoxide (DMSO). The absorbance was determined using a microplate reader (Tecan) at a test wavelength of 570 nm (reference wavelength 630 nm). The quantity of formazan product in association with the intensity of absorbance was directly proportional to the number of cultured living cells.

2.4. Measurement of cell proliferation (BrdU assay)

Cell proliferation was determined using the BrdU incorporation-ELISA assay (Roche, Swiss). At the indicated time points, cells were incubated with 10 mg/mL BrdU for 4 h before being fixed with FixDenat solution. The fixed cells were further treated with anti-BrdU-POD working solution, and rinsed with washing solution before substrate solution was added. The absorbance at 370 nm (reference wavelength at 492 nm) was read using an ELISA plate reader (Tecan).

2.5. Western blots

Western blots were performed as described elsewhere with minor modification [18]. Briefly, siRNA-treated C2C12 cells were harvested and homogenized in RIPA buffer (Cell Signaling Technology) with the protease and phosphatase inhibitor cocktail (Sigma–Aldrich). The protein concentration was determined using the protein assay dye reagent (Bio-Rad). Protein lysates were resolved on 10% SDS–PAGE gels using standard procedures. Anti-palladin (ProteinTech), anti-gapdh (Novus) antibodies were used for Western blots. Densitometric analysis was performed using the ImageJ software (NIH, Bethesda, MD).

2.6. RNA isolation and quantitative realtime polymerase chain reaction (q-PCR)

Total RNA was purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by use of a transcription first strand cDNA synthesis kit (Roche). For q-PCR, synthesized cDNA and SYBR green master-mix were run on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) with the following primer pairs: for palladin 90 kDa, 5′-CCAGGGAGCCACAAAGGAAGAC-3′; 140 kDa, 5′-TGCTGCCTGTGCATTTTCCC-3′; α-actinin, 5′-GCCTTGGACTCTGTGCCCTCA-3′; gapdh, 5′-CAGCAACTCCCACTCTTCCACC-3′. Gapdh was used as an internal loading control. Relative gene expression was determined using the ΔCT method.

2.7. Microscopy and differentiation assays

C2C12 myoblasts were grown on 0.2% gelatin coated glass coverslips to 60% confluence in GM. After siRNA transfection for 24 h, the medium was replaced with DM for 48–72 h. The differentiation assay was performed as described elsewhere with some modification [27]. Cells were harvested, fixed with 4% paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 and blocked with 1% (w/v) bovine serum albumin in phosphate-buffered saline. Palladin was visualized with murine palladin antibody (1:100; ProteinTech) and anti-mouse FITC-conjugated secondary antibody. The myosin heavy chain (MHC) was visualized with murine MHC antibody (1:500; Genetex) and anti-mouse FITC-conjugated secondary antibody. Actin was visualized with TRITC-conjugated phalloidin. Cell nuclei were counterstained with DAPI. Photographs were taken with an Eclipse Ti-E epifluorescence microscope (Nikon). For phase-contrast images, palladin-knockdown C2C12 and C2C12 control cells were grown on 6-well plates and captured every day.

2.8. Cell migration assay

C2C12 were seeded on separated inserts according to the manufacturer's instructions (ibidi, Germany) and transfected with siRNA for 24 h. The inserts were then carefully removed to release the wounds. Cells coverage to close the wound were photographed with a 10× objective at four preselected time points (0, 4, 8 and 12 h) using an optical microscope and the full-filling gap areas were evaluated by TScrash analysis software [28] and plotted for statistical analysis. The experiment was independently performed five times.

2.9. Statistical analysis

All experimental data were normally distributed and are expressed as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test to establish significance between data points. The values of $p \leqslant 0.05$ (*) and $p \leqslant 0.01$ (**) were considered statistically significant.

3. Results

3.1. Palladin expression was significantly depleted by siRNA in C2C12 myoblasts

The expression of the palladin isoforms during the transition from proliferation to early myotube differentiation of C2C12 cells induced by serum starvation was initially examined. As shown in the control cells, the amount of the 90 kDa isoform rapidly increased whereas the amount of the 140 kDa isoform was slightly decreased upon myogenesis in C2C12 myoblasts (Fig. 1B and C). These results are consistent with previously published reports on C2C12 myogenesis [18]. To explore the possible involvement of palladin during *in vitro* myogenesis, C2C12 cells were depleted of palladin with specific siRNA. Four siRNAs against different portions of the 90 kDa-palladin mRNA (Supplementary Fig. 1), that are common to all palladin isoforms were designed and assessed for silencing efficiency to find the most effective one [12]. Western blots showed that the expression of 90 kDa and 140 kDa-palladin were significantly reduced by a 48 h treatment of siRNAs (Supplementary Fig. 2A and B). The pooling of two siRNAs gained palladin knockdown efficiency (Supplementary Fig. 2A and B). Thus, these siRNAs had the ability to repress palladin expression in C2C12 cells and were suitable for the further experiments. The siRNA1 was chosen for the subsequent inhibitory experiments based on its robust capacity to decrease palladin expression in C2C12. Introduction of siRNA1 (siRNA), but not si-scrambled (Crtl), in proliferating C2C12 cells resulted in marked reduction of palladin transcripts up to 24 h post-transfection (Fig. 1A). Consistently, depletion of palladin protein was also observed in siRNA-treated cells using Western blots (Fig. 1B and C) and immunofluorescence (Fig. 1D). The knockdown effect was maintained for 3–4 days after siRNA transfection, but diminished after 6 days (data not shown).

3.2. Down-regulation of palladin enhanced C2C12 vitality and proliferation

Palladin is considered as an architectural protein and up-regulated during differentiation (Fig. 1A and B). It is speculated that palladin may be involved in cell surviving. To investigate whether palladin plays a role in myoblast survival, cell viability was examined using the MTT assay. A robust and significant increase of cell survival was observed at 48 h after the switch to low serum in siRNA-treated cells compared to control (Fig. 2A). Similarly, the treatments with other siRNAs showed a higher of survival rate than scrambled siRNA-treated C2C12 (Supplementary Fig. 3). Next, it

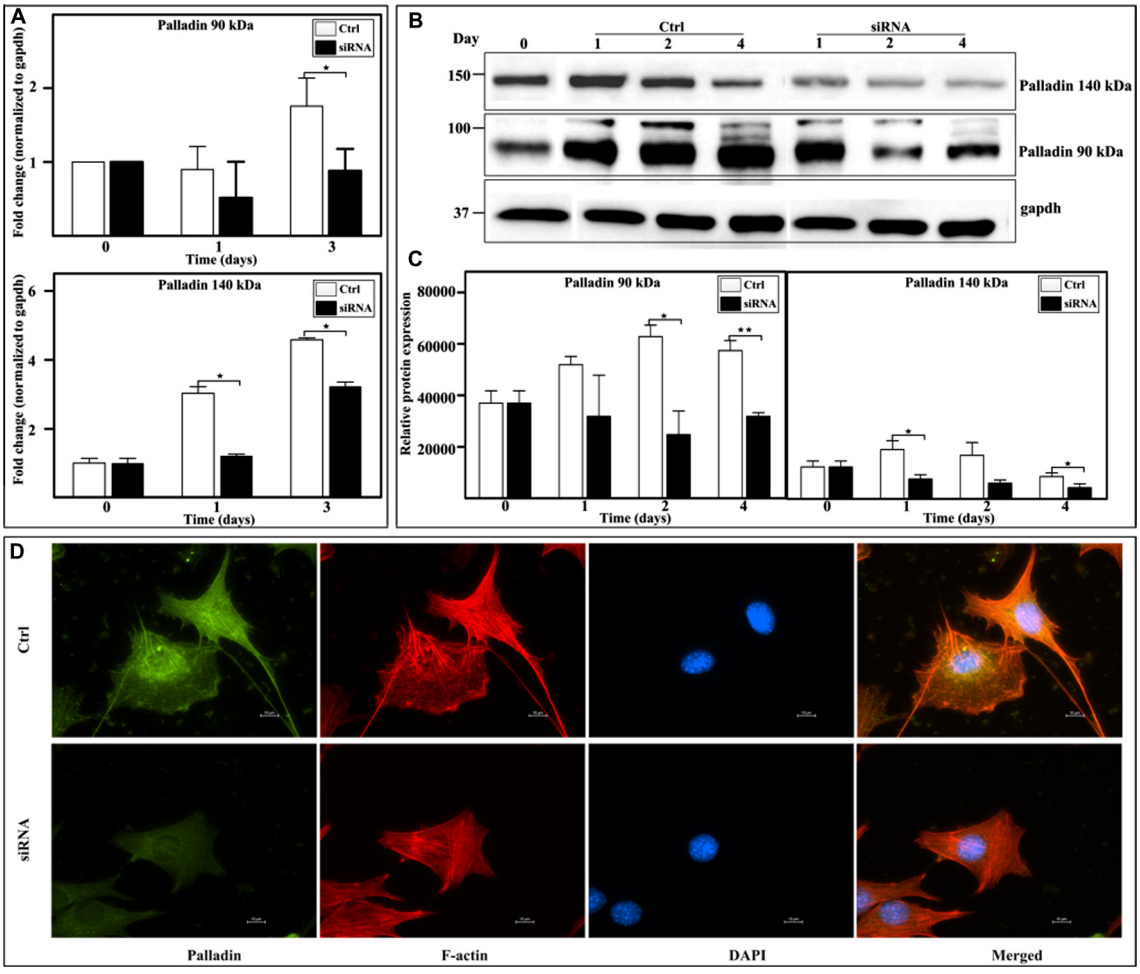

Fig. 1. Reduction of palladin expression in C2C12 myoblasts mediated by siRNA. (A) Down-regulation of palladin mRNAs in C2C12 transfected with siRNA was determined by q-PCR. C2C12 cells were transfected with palladin or scrambled siRNA at day 0 and shifted to DM 24 h post-transfection (day 1). Samples were collected at day 0, 1 and 3 after siRNA transfection. (B) Down-regulation of 90- and 140 kDa-palladin in C2C12 transfected with siRNA was determined using Western blots. Total protein was extracted at day 0, 1, 2 and 4 after siRNA transfection. Equal loading was confirmed by reprobing the blots with gapdh. (C) Densitometry of palladin expression from B. (D) Down-regulation of palladin in C2C12, detected using immunofluorescence assays. Cells were immunostained with anti-palladin (green), phalloidin-actin (red) and DAPI (blue). All data represent of at least three independent experiments. Values are presented as the mean ± SD (* indicates statistically significant difference from control cells, $^{*}p < 0.05$; $^{**}p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

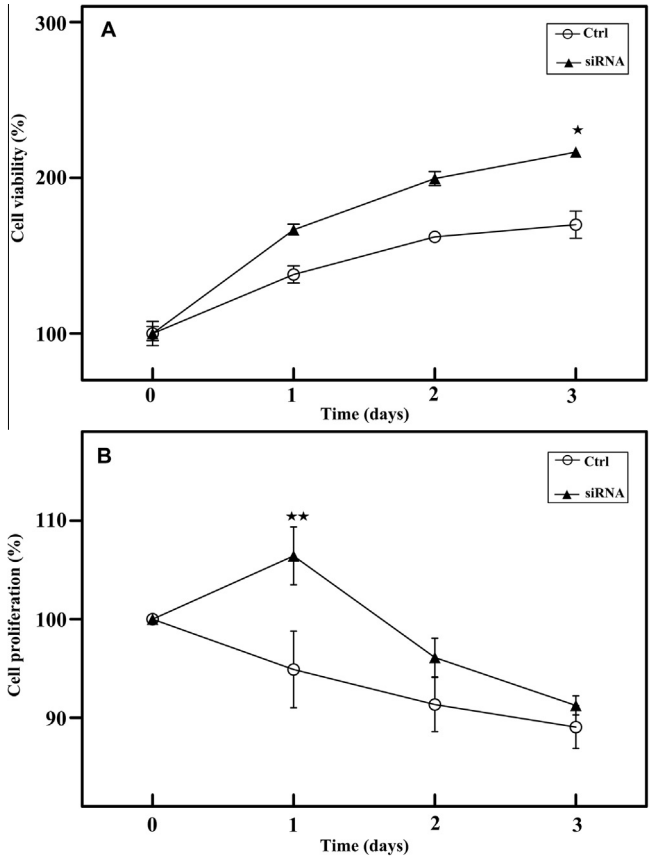

Fig. 2. Effect of palladin knockdown on viability and proliferation of C2C12 myoblasts. siRNA-mediated knockdown of palladin results in an increase in C2C12 viability and proliferation. C2C12 were transfected with either palladin-siRNA or scrambled-siRNA for 24 h and then induced to differentiate. (A) Cell viability was determined using plate-based MTT assay. (B) Cell proliferation was determined using BrdU incorporation assay. The data were evaluated from three independent experiments. Values are presented as the mean ± SD (*indicates statistically significant difference from control cells, $^{**}p < 0.01$).

was examined whether knockdown of palladin interferes with C2C12 proliferation. BrdU incorporation was employed as a measure of DNA synthesis after siRNA transfection. In a time course experiment, cell proliferation was reduced in C2C12 cells treated with siCrtl by low serum (Fig. 2B). In contrast, transient depletion of palladin-induced C2C12 proliferation after 24 h of transfection (Fig. 2B) was observed, suggesting an increased proliferation ability of palladin siRNA-treated cells compared to that of scrambled siRNA-treated cells.

3.3. Palladin repression reduced myoblast migration

It has been reported that palladin regulates the cellular migration of non-muscle cells through actin remodeling [16,20,29]. Since actin-cytoskeleton rearrangement is critical for myoblast mobility and alignment, it is supposed that palladin possibly affects myoblast motility. The effect of palladin reduction on C2C12 migration was examined using a scratch-wound healing assay (Fig. 3A). Migration results show that palladin-depleted myoblasts produced approximately a 10% decrease in the migration index after 12 h of cell damage induction (Fig. 3B). Hence, this suggests that palladin promoted the migration of C2C12 cells.

3.4. Palladin knockdown stimulated in vitro C2C12 differentiation

To determine the potential role of palladin in myoblast differentiation, C2C12 myoblasts transfected with palladin siRNAs or scrambled siRNA were induced to differentiate, and their differentiation status was assessed by measuring the number of MHC-positive cells using immunostaining (Fig. 4B). At 72 h of differentiation, more C2C12 transfected with palladin siRNA formed MHC-positive cells than those transfected with scrambled siRNA (Fig. 4B and C). In addition, palladin-knockdown cells were substantially elongated and spindle-shaped in appearance relative to the knockdown control cells after switching to DM for 48 h (Fig. 4A). These results suggest that palladin knockdown may facilitate the differentiation of C2C12 *in vitro*.

As aforementioned, several studies suggested that palladin plays important roles in the regulation of actin-cytoskeleton [15,16,30]. In addition, the binding of palladin to scaffold protein α-actinin is essential for normal cell motility [30]. Therefore, the expression of α-actinin in palladin-knocked down C2C12 was examined. It was found that despite efficient knockdown of palladin, no significant changes occurred in α-actinin transcripts (Supplementary Fig. 4). Collectively, knockdown of palladin decreased

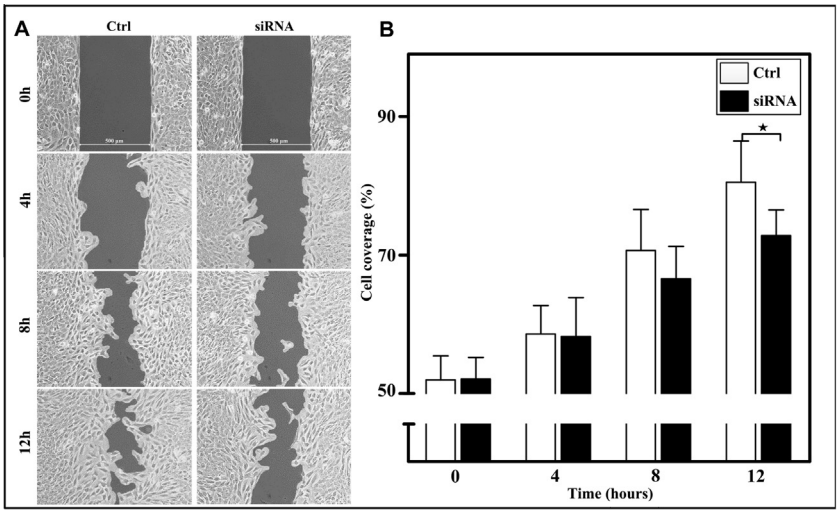

Fig. 3. Knockdown of palladin inhibited C2C12 migration. Palladin-depleted myoblasts displayed a decrease in the migration index after 12 h of cell damage induction. The results are plotted from three independent experiments. (A) The progression of cell migration or coverage of control and palladin-knockdown C2C12 was recorded under an optical microscope and subjected to statistical analysis in (B). The data were evaluated from five independent experiments. Values are presented as the mean ± SD (* indicates statistically significant difference from control cells, $^{*}p < 0.05$).

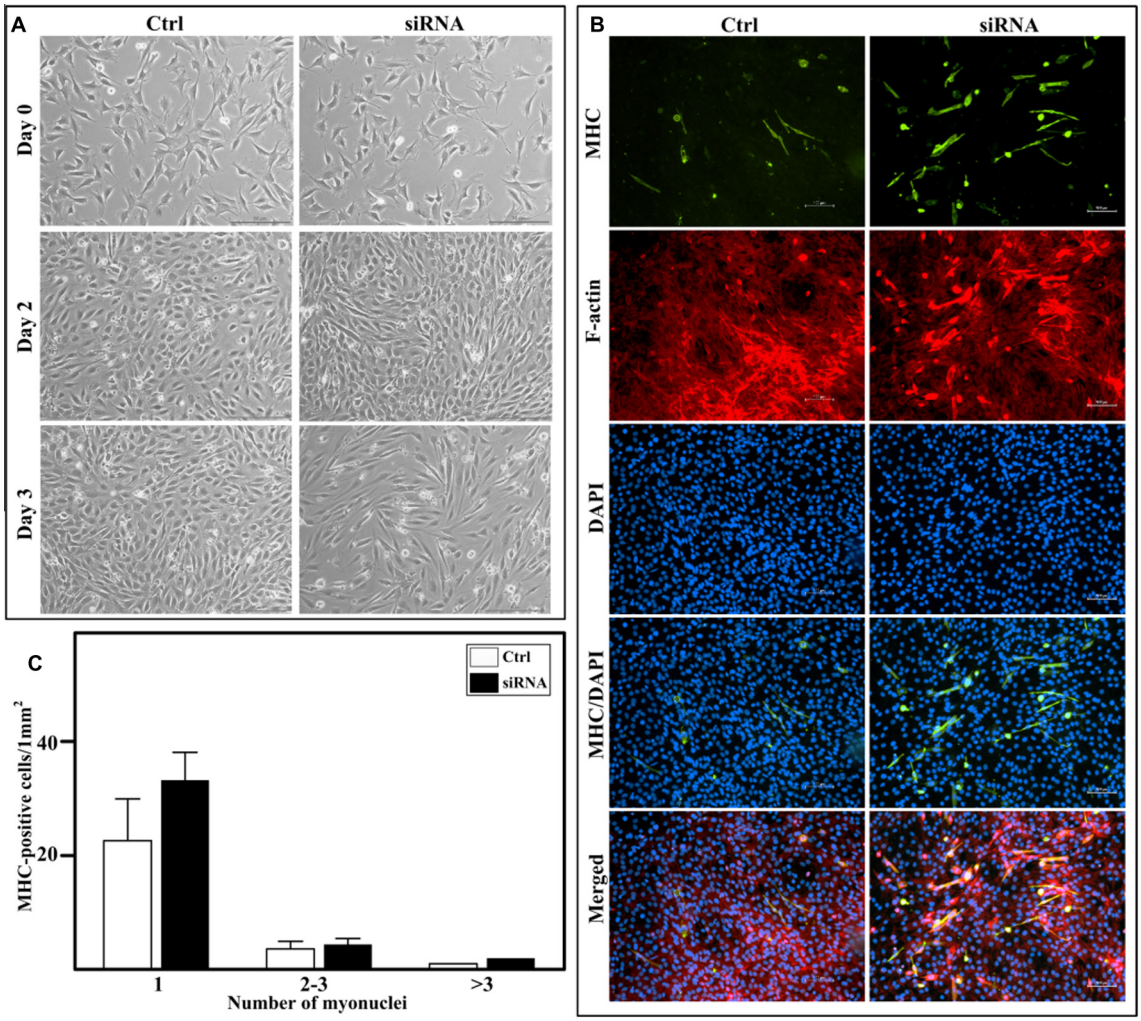

Fig. 4. Palladin silencing promoted early myogenesis *in vitro*. Myoblast differentiation and palladin knockdown. (A) Phase contrast images of C2C12 cells which had been transfected with palladin or scrambled siRNA and then shifted to DM. Note that palladin-knockdown cells showed spindle shape 2 days after switching to low serum media. (B) Cells at day 3 of differentiation were immunostained with anti-MHC (green), phalloidin-actin (red) and DAPI (blue). (C) Myotube differentiation index at day 3 of differentiation. The differentiation index was calculated using MHC-positive cells as in (B). There were fewer MHC-positive myocytes in scrambled knockdown cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

myoblast migration, but promoted C2C12 survival, proliferation and *in vitro* differentiation.

4. Discussion

It is commonly accepted that skeletal muscle differentiation is associated with dramatic cytoskeletal changes. Cytoskeletons and their related proteins are well known to be involved in the intracellular signal transduction and cytoskeleton re-organization that could be necessary for myoblast proliferation, fusion and migration to form myotubes. Palladin is a cytoskeleton-associated protein that interacts with many proteins that are required for cell mobility [20,21,31], intracellular protein trans-localization [15,32] and cytoskeleton rearrangement. Herein, an RNAi strategy was used to explore the role of palladin in *in vitro* myogenesis. The 90 kDa and 140 kDa-palladin isoforms were decreased at both protein and mRNA levels. It was observed that knockdown of palladin in proliferating myoblasts increased their viability, proliferation and differentiation. On the other hand, palladin knockdown decreased C2C12 migration. These results are consistent with previous studies in which palladin promoted cell migration [16,20,29,31,33].

In this work C2C12 myoblasts were cultured in DM to induce myogenic differentiation. Regarding vitality, it was found that knockdown of palladin increased cell viability, as evidenced using the MTT assay. Although the MTT assay is used to measure the "cell growth", this method actually measures changes in mitochondrial activity than cell proliferation. Besides, during muscle development, undifferentiated or partly differentiated cells are removed from cultures by apoptosis [34]. Therefore, the increased MTT value in the palladin-inhibited C2C12 population might also be caused by the apoptotic decreasing in myocytes. In agreement with our surmise, Zhou et al. reported that palladin promotes apoptosis through modulation of the subcellular localization of integrin-linked-kinase-associated phosphatase in HEK293 cells [35]. A study is currently underway to determine if our observed MTT increase is due to an apoptotic-resistant phenotype or an increase in mitochondrial activity.

Our experiments show that palladin depletion in C2C12 myoblasts impairs cell migration but promotes differentiation. Because cell–cell communication is needed for myogenesis induction [36], it is speculated that the cell migration decrease in palladin-depleted C2C12 reduced the changes of cell–cell contact, and hence halted myoblasts at the proliferation stage, resulting in interfering

terminal differentiation. However, a reduction of palladin resulted in an increase of C2C12 viability and proliferation, and may thus enhance the cell number in the knockdown group. This event increases the occasion for cell–cell contact that may compensate for the decrease of myoblast migration. Indeed, decreasing the migration in the knockdown group did not impair their differentiation.

Our results demonstrate that palladin mediates myoblast proliferation and migration during myogenic differentiation. Under myogenic differentiation conditions, C2C12 cells turn on a number of muscle-specific genes, including myogenin and MHC. It was found that the palladin-knockdown cells displayed elongated, spindle-shaped myocytes earlier than did the control group (Fig. 4A-Day 3). Moreover, a higher number of MHC-positive cells were observed in palladin-depleted cells than in the control group. This suggests that knockdown of palladin facilitated myoblast differentiation. Thus, palladin may play a negative role in the regulation of early myogenesis.

This study found that palladin is as an attractive target to study myogenesis. When palladin was knocked-down in C2C12 myoblast *via* siRNA, an obvious alteration of cell proliferation and migration was observed, which might affect myogenic differentiation. Regrettably, siRNA has disadvantages, such as variableness, incompleteness of knockdowns and short duration inhibition. Therefore, it is difficult to maintain the silencing efficiency during the long process of differentiation. Thus, the authors aim to create stable C2C12-harboring sh-palladin to overcome siRNA disadvantages to investigate the contribution of palladin protein in skeletal muscle differentiation.

In conclusion, our data suggest that palladin has the ability to perform some early steps of differentiation such as promoting myoblast migration, inhibiting myocyte survival, proliferation, and differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.143.

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