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Original Research

PDLIM2 Promotes Osteogenesis of Adipose-Derived Stem Cells by Modulating β-Actin-Dependent Wnt/β-Catenin Signaling

Yulian Shi[1,†], Tingyu Fan[1,†], Rongmei Qu[2,†], Guosheng Wu[1], Xiangtian Li[3], Jiaxuan Liu[1], Ziyi Feng[1], Haopeng Wu[1], Jun Ouyang[1,*], Jingxing Dai[1,*]

[1]Guangdong Provincial Key Laboratory of Digital Medicine and Biomechanics & Guangdong Engineering Research Center for Translation of Medical 3D Printing Application & National Virtual & Reality Experimental Education Center for Medical Morphology (Southern Medical University) & National Key Discipline of Human Anatomy, School of Basic Medical Sciences, Southern Medical University, 510515 Guangzhou, Guangdong, China
[2]Guangzhou HKUST Fok Ying Tung Research Institute, 511458 Guangzhou, Guangdong, China
[3]The Second Clinical Medical College, Southern Medical University, 510280 Guangzhou, Guangdong, China
*Correspondence: jouyang@smu.edu.cn (Jun Ouyang); daijx@smu.edu.cn; daijx2013@163.com (Jingxing Dai)
[†]These authors contributed equally.
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Abstract

Background: Bone tissue possesses strong regenerative potential, but large segmental bone defects often exceed its intrinsic capacity for self-healing. Human adipose-derived stem cells (hASCs) offer a promising cellular basis for bone regeneration owing to their robust osteogenic differentiation potential. However, the role of PDZ and LIM domain 2 (PDLIM2) play in hASC osteogenesis and its relationship with the Wnt/β-catenin signaling pathway remain poorly understood. **Methods:** Western blotting, immunofluorescence staining, Alizarin Red S staining, alkaline phosphatase activity assays, wound healing assays, and Transwell assays were employed to assess osteogenic differentiation, osteogenic capacity, and cellular migration. Lentivirus-mediated PDLIM2 knockdown, cytochalasin D (CytoD) intervention, microfilament stabilizer treatment, and β-catenin activator treatment were used as a means of validating the regulatory mechanism. **Results:** hASCs exhibited the ability to undergo osteogenic differentiation. During this osteogenic process, significant increases in PDLIM2, β-actin, and β-catenin expression were observed, with PDLIM2 expression reaching 2–3 times that of the control group on day 14, whereas β-actin and β-catenin levels peaked on day 7 (1.5–2 times those in the control group). Lentivirus-mediated PDLIM2 knockdown reduced β-actin expression by approximately 5-fold and β-catenin expression by approximately 1.7-fold, accompanied by cytoskeletal disorganization and impaired osteogenic differentiation. Actin microfilament integrity was found to regulate osteogenic marker expression and alkaline phosphatase activity, while CytoD treatment suppressed β-actin and β-catenin expression to approximately 50% of control levels and inhibited osteogenic differentiation. Knocking down PDLIM2 attenuated the responsiveness of hASCs to microfilament stabilizers and β-catenin activators. **Conclusions:** This study demonstrates that PDLIM2 may be functionally associated with β-catenin nuclear translocation, potentially through the remodeling of the actin cytoskeleton. These findings provide preliminary mechanistic insight and warrant further validation.

Keywords: PDLIM2; human adipose-derived stem cells (hASCs); osteogenesis; cytoskeleton; β-catenin

1. Introduction

Large bone defects resulting from trauma, infection, or oncologic resection frequently exceed the capacity of bone tissue for spontaneous healing, thereby requiring surgical intervention [1,2,3]. However, these interventions may lead to complications such as infection and bleeding [4]. Rapid advances in the field of regenerative medicine have brought innovative treatment models to clinical practice, with the stem cell-based promotion of bone regeneration holding particular therapeutic promise. Adipose-derived stem cells (ASCs) exhibit a capacity for multipotent differentiation, giving rise to a range of progeny cell types [5,6,7]. Importantly, human ASCs (hASCs) can be harvested readily, and they demonstrate paracrine activity, rendering them suitable for a broad range of wound healing, bone regeneration, and related applications [8,9]. They are widely viewed as a promising source of seed cells for bone tissue engineering. While hASCs offer great therapeutic potential, the mechanism through which they differentiate into osteoblasts is still incompletely understood. Efforts to study the molecular basis for hASC osteogenesis can thus offer valuable insight into their potential applications while providing a strong foundation for their use in the clinic. In addition to chemical stimulation, cells are exposed to a range of mechanical stimuli within the body [10], which the exposed cells convert into biochemical signals that trigger a series of intracellular reactions [11]. The osteogenic process reshapes the capacity of cells for proliferation, migration, and differentiation. Notably, there is evidence that the application of cyclic tensile loading can stimulate the expression of osteogenic factors, thus stimulating stem cells to develop into osteogenic cells [12,13].

Bioinformatics analyses have revealed a notable rise in the expression of PDZ and LIM domain 2 (PDLIM2) during the osteogenic differentiation of hASCs. PDLIM2, also known as Mystique or SLIM, is a PDLIM family member that regulates cell proliferation, migration, and differentiation [10,14]. The human *PDLIM2* gene is located on chromosome 8p21.2 [15]. Studies have shown that PDLIM2 can indirectly or directly promote interaction with the cytoskeleton by regulating the activity of important transcription factors, including nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription (STAT), and β-catenin. Abnormalities in its function may lead to tumorigenesis and oncogenic progression [10]. PDLIM2 has also been reported to play a significant role in a range of diseases. In liver cancer, it can inhibit malignant tumor progression by regulating the activity of β-catenin [16]. In one report, WD Repeat and SOCS Box Containing protein was found to inhibit the expression of PDLIM2 by promoting the ubiquitination and degradation of Kinesin Family Member 15 through a process that accelerates the accumulation of liver fat and the development of liver cancer. The Wnt/β-catenin pathway is a key regulatory pathway in. PDLIM2 can also affect the level of inflammation and bone damage in periodontitis by modulating signaling pathways, including the NF-κB and Wnt (Wingless-Type MMTV Integration Site Family)/β-catenin pathways [17].

The cytoskeleton is an integral component of cells. It plays a significant role in maintaining internal cellular structural integrity, relaying signals between the inside and outside of cells, and regulating osteogenic differentiation signaling pathways [18]. The cytoskeleton is composed of microfilaments, intermediate filaments, and microtubules [19]. Microfilaments are primarily involved in cell movement, morphological changes, and mechanical induction. Microfilament polymerization plays a key role in the osteogenic differentiation process [20]. Cytochalasin D is a well-established disruptor of the actin cytoskeleton that can specifically block actin filament polymerization, thereby affecting the osteogenic differentiation of cells. The Wnt/β-catenin pathway is a key regulatory pathway involved in osteogenic differentiation. When Wnt ligands bind to their cognate receptors, β-catenin accumulates and translocates to the nucleus, where it activates the osteogenic transcription factor Runt-related transcription factor 2 (RUNX2), promoting the differentiation of mesenchymal stem cells toward the osteoblast lineage while suppressing adipogenic differentiation. Activation of the canonical Wnt/β-catenin signaling pathway effectively facilitates bone defect repair and accelerates the healing of bone injuries [21]. Dysregulation of this pathway is associated with a range of bone diseases, making it an important therapeutic target for bone regeneration. Previous studies have shown that changes in actin status can regulate the subcellular localization and signaling activity of β-catenin [22]. As an actin-binding protein, PDLIM2 may regulate cytoskeletal tension through its interaction with actin, thereby influencing osteogenesis. Therefore, we hypothesize that PDLIM2 may affect β-catenin nuclear translocation by modulating actin polymerization status, ultimately influencing osteogenic differentiation.

Most research focused on PDLIM2 to date comes from the field of oncology, whereas its mechanistic role in the regulation of hASC osteogenic differentiation remains poorly understood. Addressing this gap in knowledge is crucial to inform the development of bone regeneration-focused therapies. In this study, hASCs were utilized as an experimental model and were treated with cytochalasin D to disrupt their microfilament networks. In order to clarify how these microfilaments influence hASC osteogenesis, Western blotting, alkaline phosphatase staining, and immunofluorescence staining were conducted. PDLIM2 expression was also knocked down via lentiviral transduction to clarify its effects on osteogenic differentiation, and responses to microfilament polymerization agents and β-catenin activators were also assessed. Ultimately, the ability of PDLIM2 to regulate Wnt/β-catenin signaling as a means of enhancing hASC osteogenic differentiation by modulating microfilament expression and structure was evaluated.

2. Materials and Methods

2.1 Cell Source

Primary human adipose-derived stem cells (hASCs) were isolated from liposuction samples obtained with written informed consent from donors. This study was approved by the Ethics Committee of Southern Medical University (Approval No. Nan Yi Lun Shen [2024] No. 25). Cells were cultured at 37 °C in a humidified 5% CO_2 incubator. The cell lines were authenticated by the flow cytometry technique. An anti-mycoplasma reagent was routinely added to the culture medium to prevent mycoplasma contamination. All cell lines used in this study were tested for mycoplasma contamination and confirmed to be negative.

2.2 Cell Culture and Osteogenic Induction

Cell culture: Human adipose-derived stem cells were isolated from three healthy female donors aged 30–40 years. Based on preoperative screening data, the donors had no apparent symptoms and were free from systemic inflammatory diseases, metabolic bone diseases, diabetes mellitus, malignant tumors, active infections and autoimmune diseases, with no history of long-term administration of glucocorticoids or immunosuppressants. Subcutaneous adipose tissue from healthy individuals was obtained, and hASCs were isolated by enzymatic digestion. Briefly, hASCs were isolated following established protocols [23,24]. When the tissue sample was fully digested, and the adipose tissue was completely dissolved, an equal amount of complete medium was added to terminate the

trypsin reaction. These tissue samples were then centrifuged at 1000 rpm for 10 minutes, the supernatant was removed, and the cell pellet was collected and resuspended in complete medium before transfer into a culture dish. After adding an appropriate amount of medium, cells were routinely cultured at 37 °C in a 5% CO_2 incubator. Culture medium was replaced every 48 h. When cells were 90% confluent, they were subcultured or cryopreserved, as appropriate [23,24]. Adipose tissue samples were provided by Nanfang Hospital's Plastic Surgery Department.

Chemical induction of osteogenic differentiation: To initiate bone cell differentiation, cells were seeded into culture plates at 7000 cells/cm^2. When they had grown to 70% confluence, standard medium was replaced with osteogenic medium containing 10 nM vitamin D_3 (V8070-1g, Solarbio, Beijing, China), 10% fetal bovine serum (10099-141, Gibco, Waltham, MA, USA), 1% antibiotics (15140-122, Gibco, Waltham, MA, USA), 10 mM β-glycerophosphate (G9422-50G, Sigma, St. Louis, MO, USA), 37.5 mg/L ascorbic acid (255564, Sigma, St. Louis, MO, USA), and 100 nM dexamethasone (D4902-25MG, Sigma-Aldrich, Shanghai, China). The culture medium was refreshed every 48 hours. Samples were taken after 0, 4, 7, and 14 days for Western blotting and alkaline phosphatase (ALP) activity assays to analyze cellular differentiation.

Induction of osteogenic differentiation through mechanical stimulation: Cells were seeded in flexible 6-well plates (FlexCell BioFlex Culture Plate, Cat. No. BF-3001C, FlexCell International Corporation, Burlington, NC, USA) at a density of 7000 cells/cm^2. When cells had grown to 70% confluence, these flexible plates were securely mounted onto the base of a tension system. Mechanical stimulation was applied using a cell culture tensile testing system (FX-5000 Tension System, FlexCell International Corporation, Burlington, NC, USA), and the entire apparatus was placed in a 37 °C 5% CO_2 incubator. A pre-programmed protocol was used to subject cells to cyclic mechanical strain at 0.5 Hz with 10% deformation for 2 hours per day. The culture medium was changed every 48 h. Samples were collected after 0, 4, 7, and 14 days for Western blotting and ALP activity assays to assess osteogenic differentiation. ALP activity was detected using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Biotechnology, Cat. No. C3206, Shanghai, China).

In appropriate experiments, cultured cells were exposed to 0.2 μg/mL cytochalasin D (MCE) dissolved in complete medium or osteogenic differentiation medium to inhibit actin polymerization. After 24 hours of routine culture, the medium was replaced with the corresponding fresh medium. After culturing for 7 days, cells were used for downstream experimental procedures.

Alternatively, cultured cells were treated with 5 μM SKL2001 (MCE) in complete medium. After 24 h of routine culture, the medium was replaced with the corresponding fresh medium, and subsequent experiments were performed after 7 days of further incubation.

2.3 Western Blotting

Cells were washed three times with PBS and then lysed on ice for 30 minutes using a total protein extraction kit (KGP250-50te, KeyGen Biotech, Nanjing, China). After centrifugation at 12,000 ×g for 15 minutes at 4 °C, the supernatant was collected, and protein concentration was determined via BCA assay. Protein samples were mixed with 5× loading buffer (FD002, Fdbio Science, Hangzhou, China) and denatured by boiling. A total of 30 μg of protein per lane was separated by 10% SDS-PAGE and then wet-transferred onto a PVDF membrane (0.22 μm, ISEQ00010, Millipore, Burlington, MA, USA). The membrane was blocked with PBS containing 5% non-fat milk (232100, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and 0.1% Tween 20 (T104863-500ml, Aladdin, Shanghai, China) for 1 hour at room temperature. Membranes were then incubated with primary antibodies diluted in Western primary antibody dilution buffer (P0023A-100ml, Beyotime, Shanghai, China) overnight at 4 °C. The primary antibodies used for this study were specific for the following targets: PDLIM2 (1:3000 Abcam, ab246868), osteopontin (1:1000 Abcam, ab69498), RUNX2 (1:1000 Cell Signaling, 12556S), β-actin (1:1000 Cell Signaling, 4970S), β-catenin (1:1000 Cell Signaling, 8480S), and GAPDH (1:5000 Bioworld, AP0063). After washing blots three times with PBST, they were incubated with HRP-conjugated secondary goat anti-rabbit IgG (H+L)-HRP (1:5000, FDR007, Fdbio Science, Hangzhou, China) or goat anti-mouse IgG (H+L)-HRP (1:5000, FDM007, Fdbio Science, Hangzhou, China) for 1 hour at room temperature. After additional washing, an ECL chemiluminescence substrate (FD8020, FDbio-Dura ECL Kit, Fdbio Science, Hangzhou, China) was used for signal development. All functional experiments were performed with three independent biological replicates. Protein expression was normalized to GAPDH as an internal reference, and data were compared between groups with Student's *t*-tests.

2.4 Immunofluorescence Staining

Cell samples were washed three times with PBS, fixed with 4% formaldehyde (DF0135, LEAGENE) for 10 minutes, and then washed three more times with PBS. After disrupting the cell membrane with 0.1% Triton-X 100 (9002-93-1, Solarbio), cells were blocked with 2% BSA and then incubated overnight at 4 ℃ with the following primary antibodies: PDLIM2 (1:50, Abcam, ab246868) and β-catenin (1:100, CST, 8480S). After washing three times with PBST, the cells were stained with Actin-Tracker-Green-488 (1:500, Beyotime, C2201S) and Alexa Fluor 488/568-conjugated antibodies (1:500, Invitrogen) and counterstained with DAPI. Fluorescence samples were captured using a confocal microscope (Carl Zeiss, LSM 880, Jena,

Germany). Fluorescence intensity data were normalized by dividing the raw values from the control and experimental groups by the mean fluorescence intensity of the control group to generate relative fluorescence intensity, followed by statistical analysis on the normalized data.

2.5 Alkaline Phosphatase Staining

Cells were washed three times with PBS, fixed with 4% formaldehyde for 10 minutes at room temperature, and then subjected to three additional washes with PBS. BCIP/NBT substrate working solution (C3206, Beyotime, Shanghai, China) was prepared and added to the cell samples, ensuring the complete coverage of all cells. Samples were then stained for 30 minutes in the dark at 37 °C. After washing with PBS, the stained cells were observed and imaged under a light microscope (Olympus, Tokyo, Japan).

2.6 Lentiviral Transduction

To explore the functional effects of *PDLIM2* knockdown on protein expression, osteogenic differentiation, and the migratory capacity of hASCs, lentivirus-mediated gene silencing was performed to stably knock down *PDLIM2* in these cells. The PDLIM2-targeting shRNA lentiviral vector (sequence No.129418) and empty control lentiviral vector used for this study were constructed by Gekai Biotechnology.

Briefly, hASCs were seeded at a density of 1.5×10^5 cells per well and incubated for 12 – 16 h. The culture medium was then replaced with fresh medium supplemented with the prepared lentivirus and a transduction enhancer reagent. In the sh-PDLIM2 group, the lentivirus was applied at a multiplicity of infection (MOI) of 30 with a viral titer of 3×10^9 TU/mL; the empty vector (NC) group was treated at the identical MOI of 30 with a viral titer of 1×10^9 TU/mL. After 14–16 h of transduction, the virus-containing medium was discarded and replaced with standard complete growth medium. At 72 h post-transduction, lentiviral transduction efficiency was quantified by detecting the proportion of GFP-positive cells via fluorescence microscopy.

2.7 Wound Healing Assay

After drawing parallel horizontal lines on the underside of 6-well culture plates, cells from the NC and sh-PDLIM2 groups were seeded into these plates at an appropriate density and allowed to grow to confluence. Then, a sterile 200 µL pipette tip was used to generate a scratch in the monolayer perpendicular to the marked lines. Cells were washed three times with PBS, and serum-free medium was then added to each well. Cell migration was observed and recorded using a microscope at 0 and 36 hours after plating.

2.8 Transwell Migration Assay

Cells were resuspended in serum-free medium, adding 5000 cells to the upper chamber of a Transwell insert (303597, Corning). Inserts were placed in 24-well plates, adding 750 µL of complete medium (high-glucose DMEM supplemented with 10% Australian fetal bovine serum, 1% penicillin–streptomycin solution, and 0.1% anti-mycoplasma reagent) to the lower chamber to establish a chemotactic gradient. After culturing cells in an incubator for 12 to 16 hours, the migrated cells were fixed with 4% formaldehyde for 10 minutes. The remaining non-migrated cells were then wiped off the upper membrane surface with a cotton swab. After washing the membrane with PBS, the remaining cells were stained with crystal violet (G1062, Solarbio) for 30 minutes. After using PBS to wash away the remaining dye, stained cells were counted using an optical microscope (Tokyo, Japan).

2.9 Statistical Analysis

All experiments were performed using hASCs isolated from three independent healthy female donors. For each donor, cells were used at passage 3–4, and all experimental conditions were tested in parallel. Data are presented as the mean ± SD from three biological replicates, with each replicate representing cells from a distinct donor. Two-tailed *t*-tests were used to analyze data in GraphPad Prism 5.0, with $p < 0.05$ as the significance threshold.

3. Results

3.1 Osteogenic Differentiation Capacity of hASCs

To validate the capacity of hASCs for osteogenic differentiation, this differentiation process was chemically induced, and cells were analyzed after 0, 4, 7, and 14 days. Western blotting revealed an increase in the expression of the osteogenic markers RUNX2 and osteopontin (OPN) with the prolongation of osteogenic induction (Fig. 1A). Alkaline phosphatase and Alizarin Red S staining intensities also increased gradually (Fig. 1B,C). Upon mechanical stimulation-based induction of osteogenic differentiation in hASCs, RUNX2 and OPN expression also progressively increased with the extension of induction time (Fig. 1D), together with the gradual enhancement of alkaline phosphatase staining (Fig. 1E). These results demonstrate that both chemical induction and mechanical stimulation can promote hASC differentiation into bone tissue.

3.2 Cytochalasin D Can Reduce PDLIM2, β-Actin, and β-Catenin Expression and Hinder hASC Osteogenic Differentiation

Next, we sought to use these hASCs to assess the regulatory impacts of microfilament organization on the expression of PDLIM2, β-actin, and β-catenin in hASCs and to determine whether these effects alter osteogenic differentiation efficiency. To that end, we treated hASCs with cytochalasin D (Cat. No.: HY-N6682, MedChemExpress,

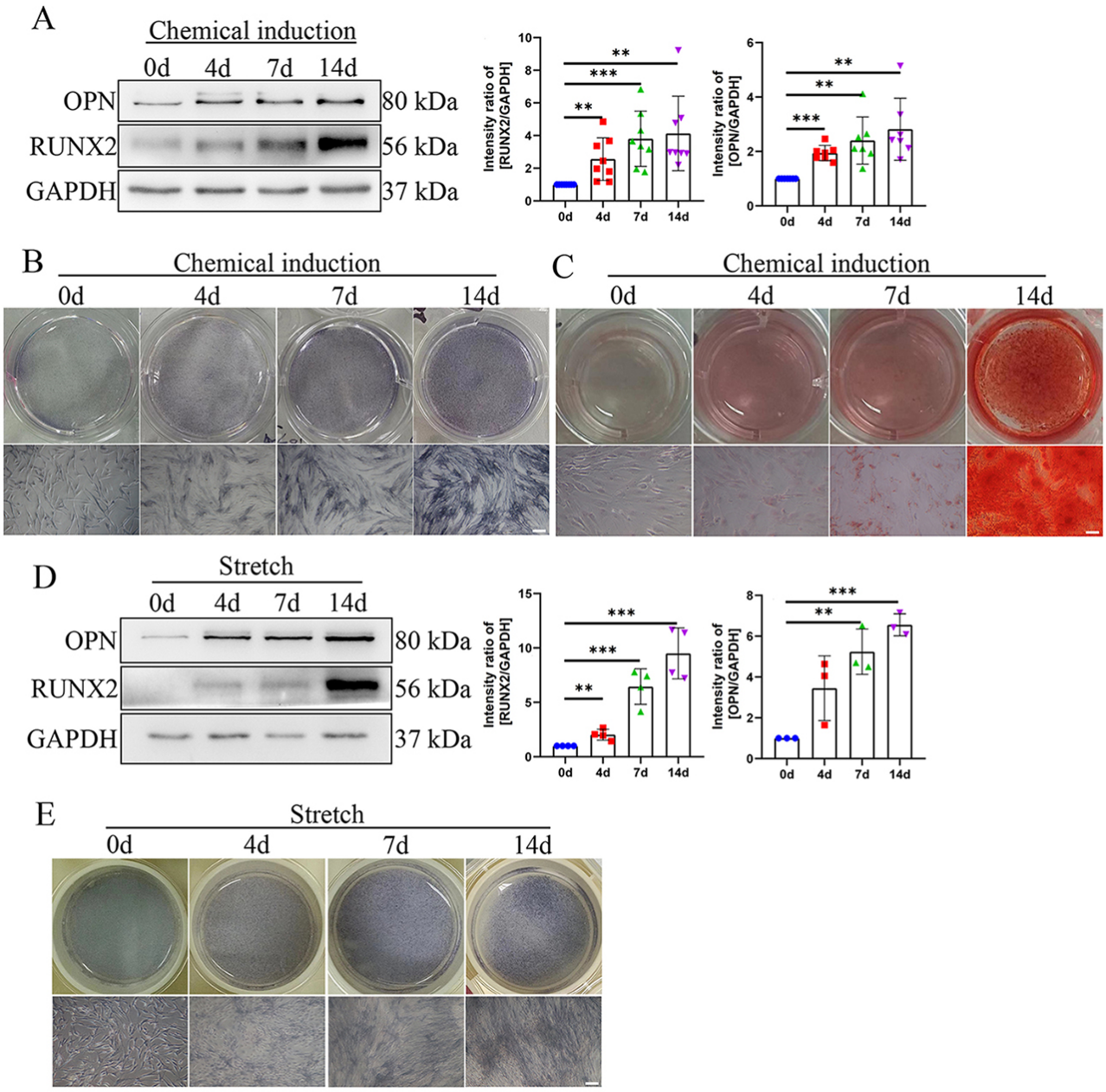

Fig. 1. Osteogenic differentiation of human adipose-derived stem cells (hASCs). (A) Western blotting results and corresponding quantification of osteogenesis-related proteins in chemically induced hASCs. (B) ALP staining results for chemically induced osteogenic differentiation in hASCs. (C) Alizarin Red S staining results for chemically induced osteogenic differentiation in hASCs. (D) Western blotting results and corresponding quantification for mechanically stimulated hASCs. (E) ALP staining images for mechanically induced osteogenic differentiation in hASCs. Scale bar, 200 μm (n ≥ 3, $^{**}p < 0.01$, $^{***}p < 0.001$). ALP, alkaline phosphatase.

USA), which led to significant reductions in PDLIM2, β-actin, and β-catenin protein levels in these cells, consistent with the marked disruption of the microfilament cytoskeletal structure. Cytochalasin D inhibited the ability of hASCs to differentiate into osteoblasts, as was further confirmed by alkaline phosphatase and Alizarin Red S staining. These results suggest that inhibiting microfilament polymerization can inhibit the osteogenesis of hASCs. The inhibition of cytoskeletal polymerization also resulted in a decrease in the expression of PDLIM2. Based on these results, we hypothesize that PDLIM2 interacts with the microfilament cytoskeleton to modulate the osteogenic differentiation potential of hASCs (Fig. 2).

3.3 hASC Osteogenic Differentiation Coincides With Changes in Microfilaments and β-Catenin

Next, the roles that PDLIM2, β-actin, and β-catenin play during the osteogenic differentiation of hASCs were examined via Western blotting to explore changes in protein expression throughout this process. Quantitative anal-

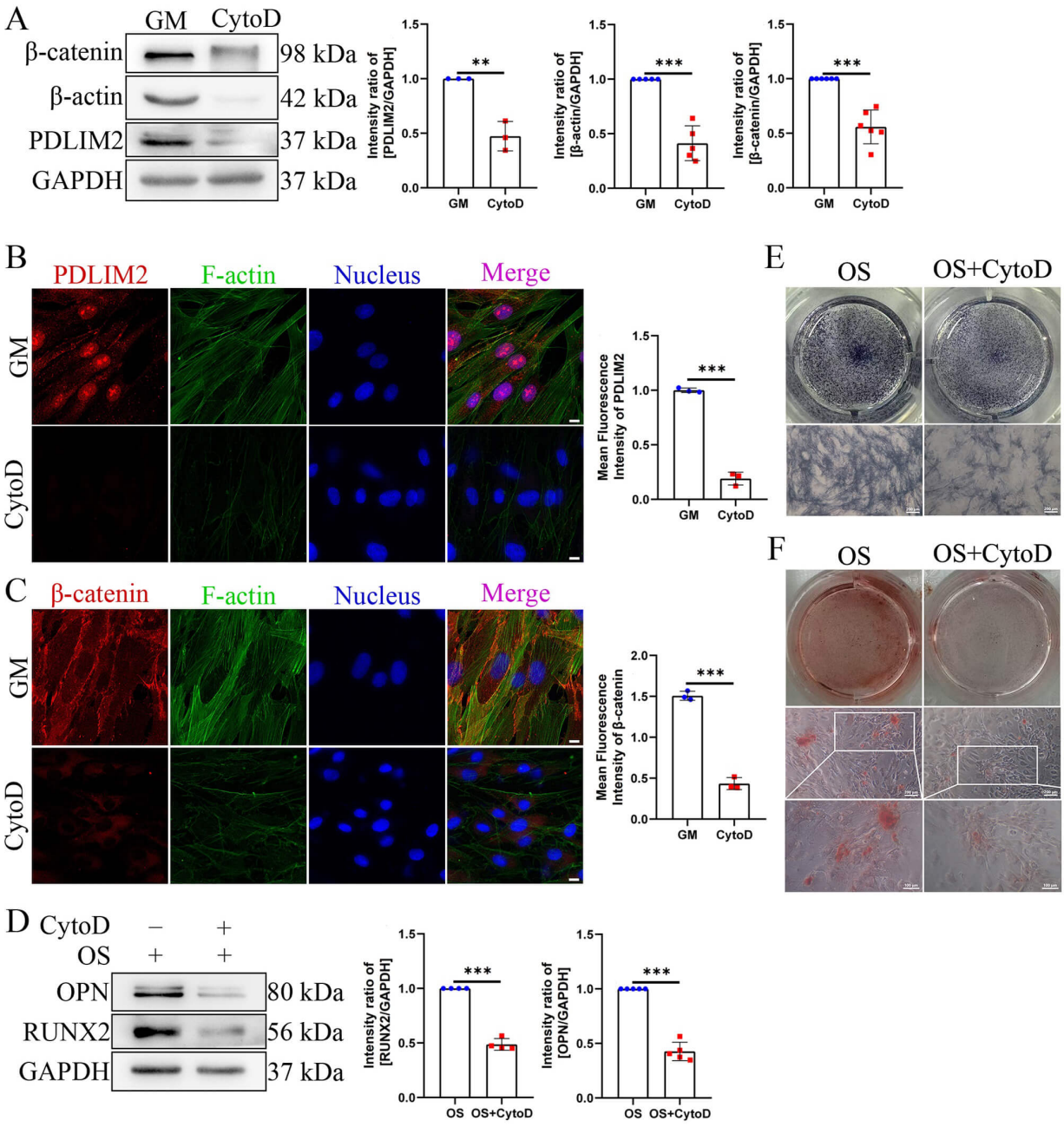

Fig. 2. Effects of cytochalasin D on the osteogenic differentiation of human adipose-derived stem cells (hASCs) and related protein expression. (A,D) Western blotting analysis and corresponding quantification of the effects of cytochalasin D on protein expression and osteogenesis. (B,C) Immunofluorescence results showing the effects of cytochalasin D on the expression of PDLIM2, β-catenin, and F-actin. (E,F) ALP and Alizarin Red S staining results for the osteogenic differentiation of hASCs treated with cytochalasin D. Scale bar, 10 μm (B,C); 200 μm (E,F); 100 μm (insets in F) ($n \geq 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). PDLIM2, PDZ and LIM domain 2.

yses revealed progressive increases in β-actin and β-catenin levels throughout the differentiation period, peaking on day 7. While these levels declined slightly on day 14, they remained significantly elevated compared with those at baseline. The expression of PDLIM2 exhibited a continuous upward trend throughout the differentiation process. Immunofluorescence staining further confirmed these results, demonstrating that osteogenic differentiation significantly upregulated PDLIM2, β-actin, and β-catenin expression, leading to cytoskeletal remodeling. These findings suggest that PDLIM2 and β-actin may play important regulatory roles in the osteogenic differentiation of hASCs (Fig. 3).

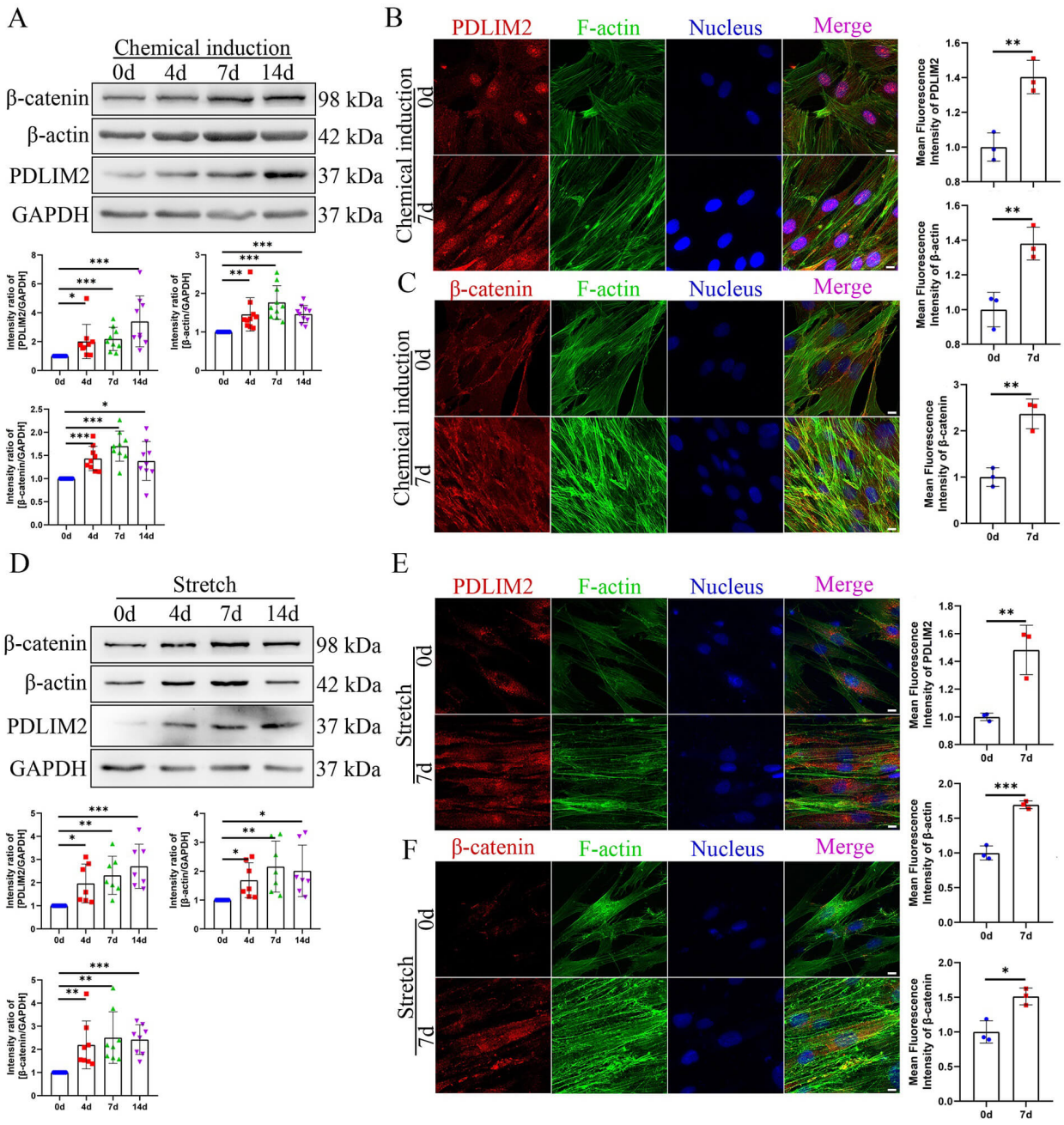

Fig. 3. Protein expression during the osteogenic differentiation of human adipose-derived stem cells (hASCs). (A,D) Expression of PDLIM2, β-actin, and β-catenin during the chemically and mechanically induced osteogenic differentiation of hASCs. (B,E) Immunofluorescence staining results for PDLIM2 and F-actin during the chemically and mechanically induced osteogenic differentiation of hASCs. (C,F) Immunofluorescence staining results for β-catenin and F-actin during the chemically and mechanically induced osteogenic differentiation of hASCs (n ≥ 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Scale bar, 10 μm.

3.4 PDLIM2 Knockdown Inhibits the Proliferation, Migration, and Differentiation of hASCs

To investigate the functional role of PDLIM2 in hASCs, lentiviral-mediated knockdown was initially performed to reduce its expression. Confocal microscopy (LSM 880, Zeiss, Germany) revealed approximately 80% transduction efficiency, and both Western blotting and immunofluorescence staining confirmed successful knockdown of this protein. Using the resulting PDLIM2 knockdown cells, we performed wound healing and Transwell migration assays. A pronounced decrease in cellular proliferation and migration was observed following PDLIM2 knockdown relative to control. To elucidate the mechanistic importance of PDLIM2 in osteogenic differentiation,

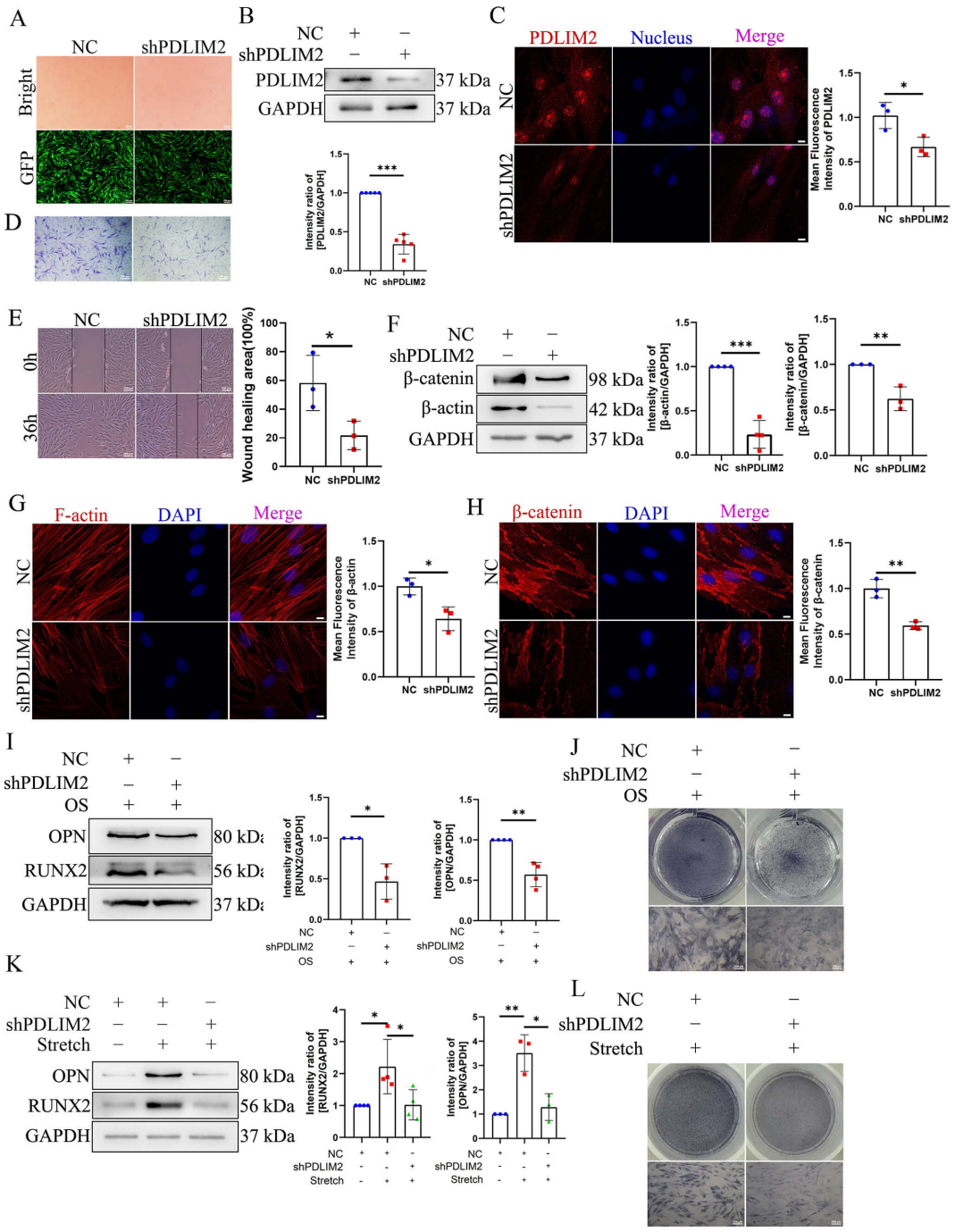

Fig. 4. Confirmation of PDLIM2 knockdown and evaluation of its effect on osteogenic differentiation. (A) GFP fluorescence images confirming PDLIM2 knockdown. (B) Western blotting confirmation of PDLIM2 knockdown. (C) Immunofluorescence confirmation of PDLIM2 knockdown. (D) Transwell migration assays following PDLIM2 knockdown. (E) Wound healing assay following PDLIM2 knockdown. (F) Western blotting analysis of the effects of PDLIM2 knockdown on β-actin and β-catenin. (G) Immunofluorescence staining for F-actin after PDLIM2 knockdown. (H) Immunofluorescence staining for β-catenin after PDLIM2 knockdown. (I) Quantification of protein levels following PDLIM2 knockdown and 7 days of chemical osteogenic induction. (J) Alkaline phosphatase staining result corresponding to PDLIM2 downregulation after 7 days of chemical osteogenic induction. (K) Quantification of protein levels following PDLIM2 knockdown and 7 days of mechanical osteogenic induction. (L) Alkaline phosphatase staining result corresponding to PDLIM2 downregulation after 7 days of mechanical osteogenic induction. Scale bar, 200 μm (A,D,E,J,L); 10 μm (C,G,H). ($n \geq 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). GFP, Green fluorescent protein.

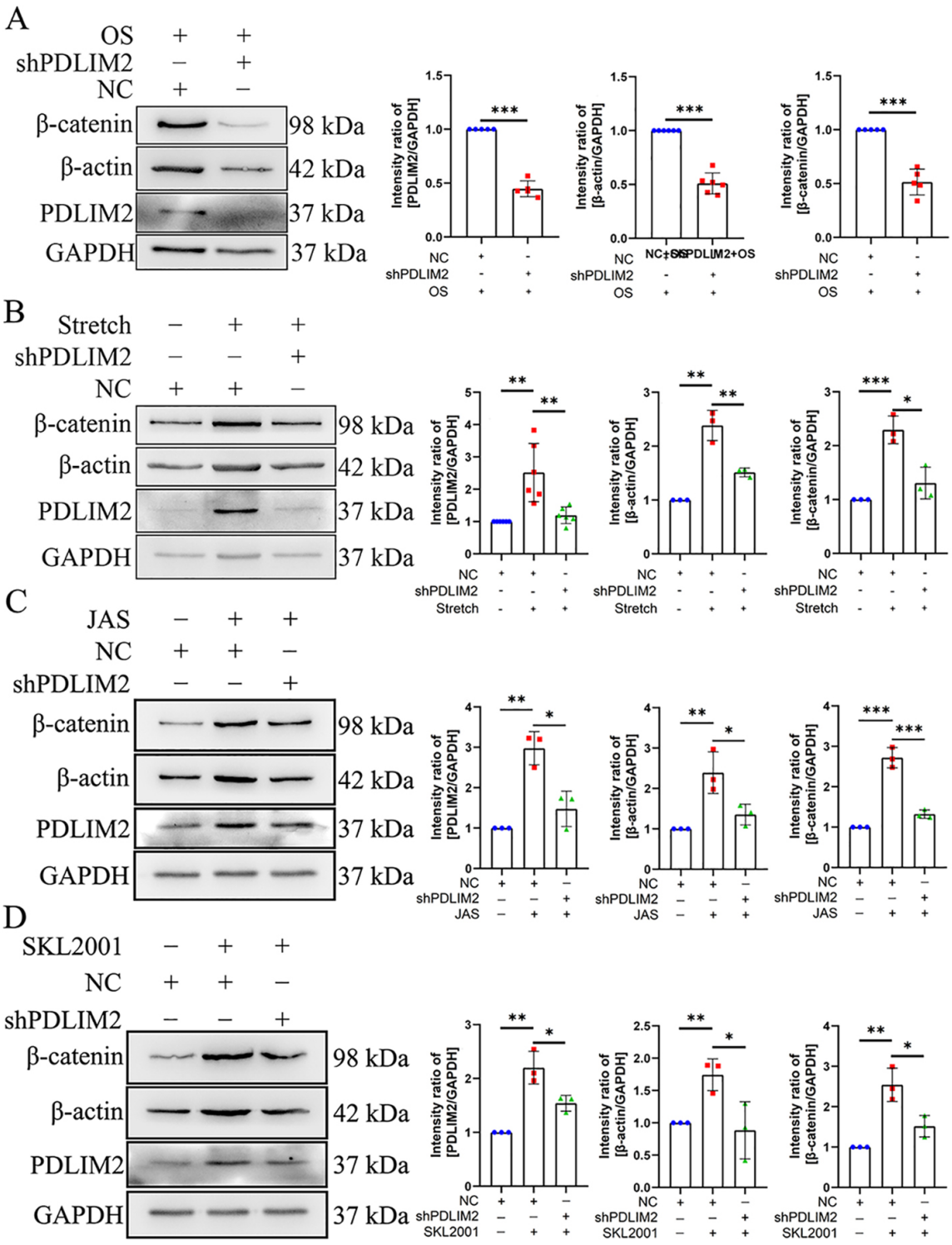

Fig. 5. Effects of PDLIM2 knockdown. (A–D) Western blotting and corresponding quantification of the expression of the indicated proteins at 7 days following (A) chemically induced osteogenic induction, (B) mechanically induced osteogenic induction, (C) microfilament polymerizer treatment, and (D) β-catenin activator treatment 7 days after PDLIM2 downregulation (n ≥ 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

we treated PDLIM2-knockdown hASCs with osteogenic induction medium (high-glucose DMEM supplemented with 5% Australian fetal bovine serum, 1% penicillin–streptomycin solution, 0.1% anti-mycoplasma reagent, 10 nM vitamin D_3, 37.5 mg/L ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate disodium salt) or subjected them to mechanical loading. Western blotting and alkaline phosphatase staining confirmed a signifi-

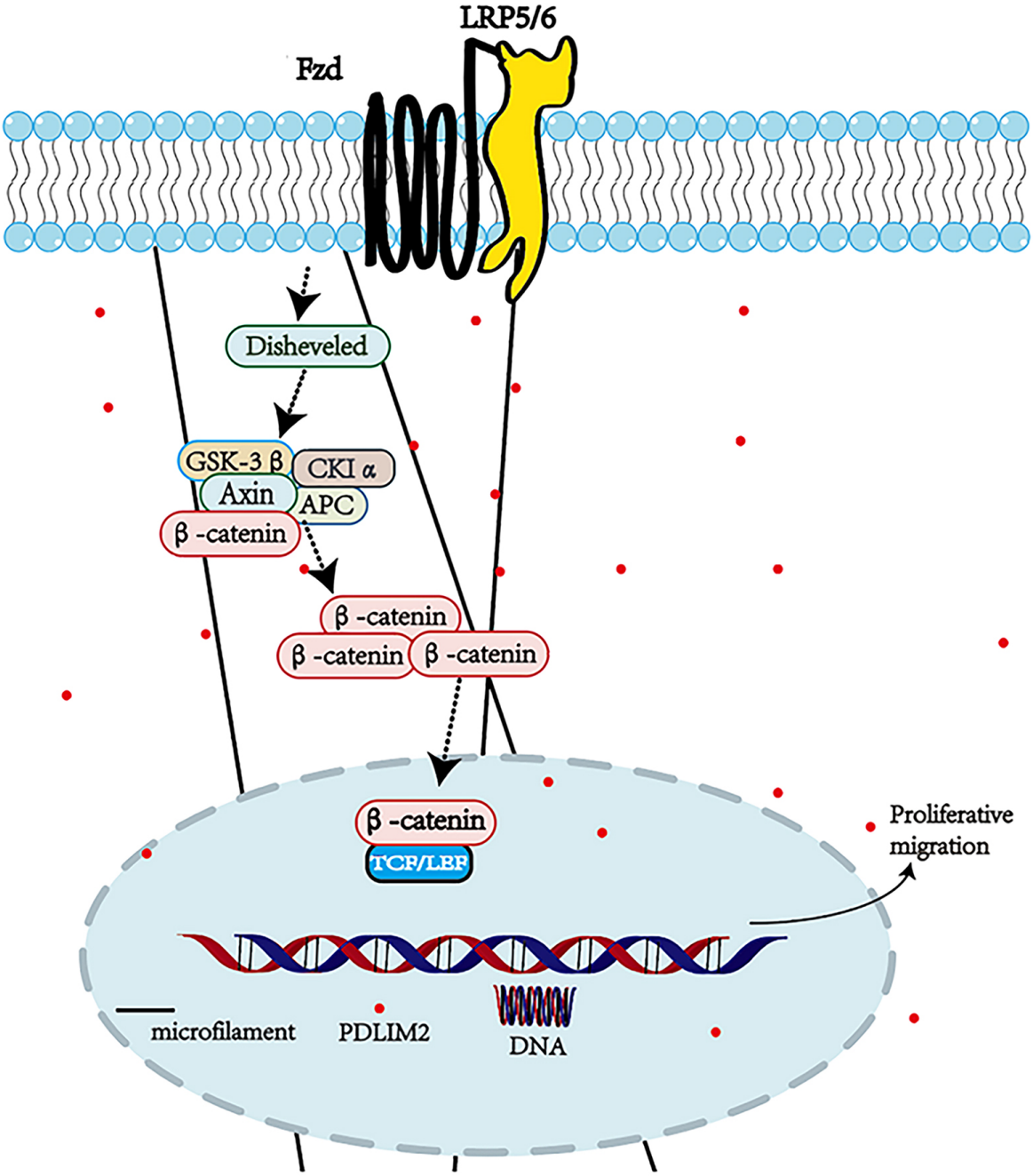

Fig. 6. This schematic presents the proposed mechanistic role of PDZ and LIM domain 2 (PDLIM2) in the osteogenic differentiation of human adipose-derived stem cells (hASCs). Our findings establish PDLIM2 as a key driver of hASC osteogenesis through its ability to orchestrate β-actin-mediated Wnt/β-catenin signaling, thus offering novel insights that may inform the development of new bone regeneration strategies. The schematic was independently redrawn using Adobe Illustrator.

cant reduction in the capacity of these cells for osteogenesis following PDLIM2 knockdown. The present results suggest that PDLIM2 expression levels are closely correlated with changes in the proliferative capacity, migratory potential, and differentiation processes of hASCs. In addition, PDLIM2 expression is associated with alterations in stem cell fate-related cellular activities (Fig. 4).

3.5 PDLIM2 Downregulation Impairs Osteogenic Differentiation and Attenuates Responses to Jasplakinolide (JAS) and β-Catenin Activation

In light of the observation that changes in PDLIM2 expression closely parallel the osteogenic differentiation of hASCs, we next examined the functional characteristics of PDLIM2 more broadly across a range of experiments. After using lentivirus-mediated gene silencing to downregulate PDLIM2 expression, cells were subjected to various

treatments, including chemical-induced osteogenic differentiation, mechanical stimulation-induced osteogenic differentiation, and Jasplakinolide (Cat. No.: J4580, Sigma-Aldrich, USA) and β-catenin activation. In these assays, PDLIM2-knockdown cells exhibit much weaker responses to all four stimuli as compared to control cells. Specifically, cells with reduced PDLIM2 expression exhibited significantly decreased production of β-actin and β-catenin at the protein level (Fig. 5). Based on these data, we hypothesized that the loss of PDLIM2, as an actin-binding protein, may lead to impaired integrity or dynamic imbalance of the actin cytoskeleton. Such actin cytoskeleton abnormalities can, in turn, affect multiple osteogenesis-related signaling pathways, potentially including the Wnt/β-catenin pathway [25]. PDLIM2 may also be involved in JAS-induced actin polymerization. In the absence of normal PDLIM2 expression, JAS cannot effectively exert its actin-stabilizing effect, thereby weakening its ability to promote osteogenic differentiation. Similarly, the abnormal actin polymerization status that results from PDLIM2 knockdown may hinder the efficient nuclear entry of β-catenin. Consistently, upon exogenous treatment with a β-catenin activator (Cat. No.: HY-101085, MedChemExpress, USA), impaired β-catenin nuclear translocation was found to coincide with reduced downstream transcriptional activity, supporting a correlative relationship between these events.

4. Discussion

When used in the context of bone regenerative medicine, ASCs offer unique benefits over bone marrow mesenchymal stem cells, primarily due to the abundance of sources from which ASCs can be readily harvested [8,9]. Under specific induction conditions, ASCs can differentiate into a range of cell types. In this study, the mechanisms through which PDLIM2 regulates the osteogenic differentiation of hASCs were explored. To that end, the mutual regulatory relationships between PDLIM2 and the actin cytoskeleton (particularly β-actin), as well as the β-catenin signaling pathway, were systematically investigated in the context of osteogenesis in an effort to establish a novel theoretical foundation and to identify relevant targets to guide bone tissue regeneration.

As the main component of the cytoskeleton, actin participates in signal transduction inside and outside cells while also serving as a central regulator of stem cell differentiation into osteoblasts by influencing the activities of crucial molecules in bone formation-related pathways [18,26]. During the course of hASC osteoblastic transformation, we observed a marked increase in PDLIM2 production. Interestingly, when we disrupted microfilaments using cytochalasin D, the capacity of these hASCs for bone formation was greatly reduced, as evidenced by decreased levels of key markers associated with bone formation. This phenomenon suggests that the polymerization state of the microfilament skeleton plays a significant regulatory role in the osteogenesis of hASCs. These results imply that the constant rearrangement of the cellular skeleton is integral to how efficiently hASCs differentiate into cells that form bone and that PDLIM2 may interact with actin in this process.

As a PDLIM family protein, PDLIM2 has been established as an important cytoskeleton-related protein that participates in the regulation of cell differentiation and various disease processes while also being closely related to the microfilament cytoskeleton [27]. In the present study, we found that knocking down PDLIM2 expression in hASCs was accompanied by the significant inhibition of cell growth and motility, decreased microfilament expression, disrupted cell morphology, reduced β-catenin levels, and impaired osteogenic capacity. These parallel changes suggest a potential correlative link among PDLIM2, microfilament organization, β-catenin expression, and osteogenic progression in hASCs. To further verify this mechanistic link, we treated PDLIM2 knockdown cells with microfilament stabilizers and β-catenin activators. We found that, while all treatments increased the levels of PDLIM2, β-actin, and β-catenin in hASCs, the observed increases in β-actin and β-catenin levels were notably smaller in cells with reduced PDLIM2 expression as compared to the control group. PDLIM2 appears to regulate key signaling pathways involved in bone formation by modulating microfilament activity, which in turn controls how hASCs grow and develop (Fig. 6). Research on PDLIM2 to date has primarily focused on the oncology field, where PDLIM2-mediated regulation of Wnt/β-catenin signaling plays a role in cancer development and progression. The Wnt/β-catenin signaling pathway is also one of the key regulatory pathways involved in osteogenic differentiation. Previous studies have shown that changes in actin status can regulate the subcellular localization of β-catenin. Therefore, we hypothesize that PDLIM2, as an actin-binding protein, may modulate actin polymerization status, thereby regulating β-catenin nuclear translocation and ultimately influencing osteogenic differentiation. Immunofluorescence results confirmed the close colocalization of PDLIM2 with microfilaments, suggesting a potential interaction. However, the precise mode of interaction between PDLIM2 and microfilaments, be it one mediated by direct binding or the actions of intermediate molecules, remains uncertain. Further research is also needed to clarify the mechanisms through which it regulates β-catenin, with potential transcriptional regulation, protein stabilization, or subcellular localization changes requiring additional experimental approaches such as colocalization and co-immunoprecipitation. The in-depth study of these mechanisms will provide a more comprehensive theoretical basis for understanding the role of PDLIM2 in the process of bone regeneration.

5. Limitations and Future Perspectives

The present results offer confirmation of the role of PDLIM2 in the regulation of the osteogenic differentiation of hASCs while also offering preliminary insight into its correlative associations with the β-catenin signaling pathway. However, the observed relationships between PDLIM2, actin remodeling, and β-catenin nuclear translocation were solely correlative and do not necessarily indicate any causal associations. Multi-level mechanistic analyses are still required, as the transcriptional regulation of β-catenin by PDLIM2 was not examined in detail, nor were any efforts made to detect PDLIM2-mediated changes in β-catenin post-translational modifications or protein stability, and the mechanisms governing the subcellular localization and nucleocytoplasmic shuttling of β-catenin remain to be further elucidated. In addition, only PDLIM2 knockdown-mediated loss-of-function experiments were performed in this study, whereas rescue and gain-of-function validation were not conducted due to experimental constraints. In addition, no *in vivo* analyses were performed to confirm these results. These limitations restrict current understanding of the PDLIM2-β-catenin regulatory network, and the molecular mechanism by which PDLIM2 regulates the osteogenic differentiation of stem cells requires further characterization.

6. Conclusion

These results indicate that dynamic changes in PDLIM2 expression are closely associated with the osteogenic differentiation of hASCs. During osteogenesis, β-actin-mediated cytoskeletal remodeling and the activity of the canonical Wnt/β-catenin signaling pathway exhibit synchronous parallel changes. These changes suggest that PDLIM2 regulates hASC osteogenic differentiation at least in part via the canonical Wnt/β-catenin signaling pathway. However, the process of osteogenic differentiation is complex and synergistically regulated by multiple signaling pathways. Rather than acting solely through the Wnt pathway, PDLIM2 may also engage in molecular crosstalk with other key osteogenic signaling pathways, including the BMP/SMAD, MAPK, Hippo/YAP-TAZ, and NF-κB pathways, to collectively govern the osteogenic fate of stem cells.

Abbreviations

PDLIM2, PDZ and LIM domain 2; hASCs, human adipose-derived stem cells; ASCs, adipose-derived stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween-20.

Availability of Data and Materials

All study data can be requested by contacting the corresponding author.

Author Contributions

Concept and design: TF, RQ, GW, XL, JD and JO; data collection and analysis: YS, JL, ZF, TF, RQ, GW, XL and HW; drafting the article: YS, TF, RQ, GW, and XL; critical revision of the article: JD and JO; study supervision: JD and JO. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental methods and protocols performed in this study were approved by the Biomedical Ethics Committee of Southern Medical University. Approval number: [2024] 25. Patients provided written informed consent for the use of samples. All experimental methods and protocols performed in this study were in accordance with the relevant guidelines and regulations (Declaration of Helsinki).

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Conflicts of Interest

The authors declare no conflicts of interest.

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