Deletion of Peroxiredoxin II Inhibits the Growth of Mouse Primary Mesenchymal Stem Cells Through Induction of the G₀/G₁ Cell-cycle Arrest and Activation of AKT/GSK3 β / β -Catenin Signaling

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Abstract. *Background/Aim:* Dermal mesenchymal stem cells (DMSCs) are pluripotent stem cells found in the skin which maintain the thickness of the dermal layer and participate in skin wound healing. *Materials and Methods:* The MTT assay was performed to detect cell proliferation and cell-cycle progression and cell-surface markers were assessed by flow cytometry. The levels of proteins in related signaling pathways were detected by western blotting assay

and the translocation of β -catenin into the nucleus were detected by immunofluorescence. Red oil O staining was performed to examine the differentiability of DMSCs. *Results:* Knockout of PRDX2 inhibited DMSC cell growth, and cell-cycle arrest at G₀/G₁ phase; p16, p21 and cyclin D1 expression levels in Prdx2 knockout DMSCs were significantly increased. Furthermore, AKT phosphorylation were significantly increased in Prdx2 knockout DMSCs, GSK3 β activity were inhibited, result in β -Catenin accumulated in the nucleus. *Conclusion:* In conclusion, these results demonstrated that PRDX2 plays a pivotal role in regulating the proliferation of DMSCs, and this is closely related to the AKT/glycogen synthase kinase 3 beta/ β -catenin signaling pathway.

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Skin has a strong capacity for repair and regeneration due to the fact that it contains various stem cells, such as epidermal stem cells, skin-derived precursors and dermal mesenchymal stem cells (DMSCs) (1, 2). DMSCs play two major roles, one is as a direct source of fibroblasts, and the other is to secrete a variety of cytokines which promote fibroblasts to produce collagen and elastin (3). Therefore, the function of DMSCs plays decisive role in skin development and wound repair (4).

Peroxiredoxin 2 (PRDX2) is widely distributed in various tissues and cells, and functions as a scavenger of reactive oxygen species (ROS) (5, 6). Our previous study showed that

Prdx2 knockout mice had significant symptoms of skin aging, which are characterized by reduced skin thickness and thinning of the dermis (7), suggesting that PRDX2 may play a regulatory role in dermal cells. We also reported that *Prdx2* knockout can induced cellular senescence of embryonic fibroblasts through ROS-dependent signaling pathway (7), however, the role of PRDX2 in the regulation of DMSC proliferation is not clear.

The Wnt signal is activated by Wnt ligand binding to a frizzled receptor (8). In the absence of Wnt ligands, the downstream signaling molecule β -catenin can be phosphorylated by glycogen synthase kinase 3 beta (GSK3 β) and then phosphorylated β -catenin is degraded by ubiquitination (9). When Wnt ligand binds to the receptor, the phosphorylation of β -catenin by GSK3 β is inhibited, which results in accumulation of β -catenin in the cytoplasm, it finally being transferred to the nucleus to induce the expression of target genes (10, 11). Therefore, phosphorylation of β -catenin and GSK3 β is a marker distinguishing the activation of the classical Wnt/ β -catenin signal (12). Previous studies have shown that PRXs play a role in cell proliferation *via* Wnt signaling. It has been reported that knockdown of PRDX2 can inhibit the growth of colon cancer cells by inhibiting Wnt signaling pathway (13), but down-regulation of PRDX5 inhibited the growth of chondrocytes through activating the Wnt signaling pathway (14). These findings suggests that PRXs may participate in cell proliferation by modulating Wnt signaling in different ways. The role of PRDX2 and Wnt signaling in the regulation of DMSC proliferation has not been elucidated.

Therefore, in this study, we used both wild-type and *Prdx2* knockout DMSCs to study the effect of PRDX2 on DMSC proliferations and molecular mechanisms, especially on activation of β -catenin signaling under normal cell culture conditions, in order to understand the regulatory function of PRDX2 in DMSC growth.

Materials and Methods

Animals. *Prdx2*^{+/+} (wild-type) and *Prdx2*^{-/-} 129/SvJ mice were provided by the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Mice were kept under the temperature at 20-22°C, the humidity 50-60% and the 12-h-dark/light cycles conditions and provided food and water *ad libitum*. The Institutional Animal Care and Use Committee approved both the animal care and experiments.

Cell isolation and cell culture. The dorsal skin of newborn wild-type and *Prdx2*^{-/-} mice was collected and spread in 3.5 cm culture dish with 2 ml 0.25% trypsin-EDTA (T/E; Solarbio, Beijing, China) for 4 h at 4°C. The subcutaneous fat and blood vessels on skin were removed using tweezers. The skin was then treated for 1 h at 37 °C in T/E to Separation of dermis and epidermis. The epidermal-free tissue were dissected into 1.0 cm² pieces and digested with 0.25% T/E for 1 h at 37°C. After that, the cells were collected by

centrifugation at 300 × *g* for 7 minutes and cultured in growth medium consisting of Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) and Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Gibco-BRL) with 2 mM L-glutamine and 1% non-essential amino acid solution (NEAA; Solarbio, Beijing, PR China), containing 10% fetal bovine serum (FBS; Solarbio) and 1% 100 U/ml penicillin-streptomycin (P/S; Solarbio). The cells were cultured at 37°C under 5% CO₂.

Detection of surface antigens. *Prdx2*^{+/+} (wild-type) and *Prdx2*^{-/-} DMSCs from passage 3 were seeded in six-well plates at density of 3×10⁵ cells/well. After cell adherence for 24 h, the cells were washed with phosphate buffer (PBS; Solarbio, Beijing, PR China) twice, permeabilized, and then immune stained with the following fluorochrome-conjugated antibodies overnight at 4°C: anti-CD44-phycoerythrin (PE), anti-CD106-PE, anti-CD14-fluorescein isothiocyanate (FITC), anti-CD34-PE and anti-CD45-FITC (BioLegend, San Diego, CA, USA). The MSC phenotypes of DMSCs were characterized by flow cytometric analysis (FACScan; BD Biosciences, San Jose, CA, USA).

Multilineage differentiation potential of DMSCs. DMSCs from passage 3 were seeded in a 3.5 cm culture dish and cultured with osteogenic differentiation medium (Solarbio) when cells reached 70% confluence. The culture medium was changed every 2 days. After 21 days of differentiation, the cells were permeabilized with 70% alcohol for 10 min and stained with 0.1% alizarin red S pH 8.3 for 7 min (Sigma-Aldrich, St. Louis, MO, USA). Finally, images were obtained by fluorescence microscopy coupled with a camera (Leica DM2500, Germany).

The double types of DMSCs from passage 3 (3 P) were seeded in 3.5 cm culture dish and cultured with adipogenic differentiation medium (Solarbio) when cells reached 70% confluence. The culture medium was changed every 2 days. After 15 days of differentiation, the cells were permeabilized with 4% paraformaldehyde for 1 h, treated with 60% isopropanol for 2 min and stained with 0.5% Oil-red O for 20 min (Solarbio). Finally, images were obtained with a DM2500 camera (Leica, Wetzlar, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2.5×10³ cells/well) were cultured in 96-well plates with growth medium at 37°C for 1, 2, 5, and 7 days. MTT (10 μ l of 5 mg/ml) was added at specific times during incubation for 4 h and formazan was dissolved using dimethyl sulfoxide (100 μ l). A micro-plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to measure the absorbance of cells at 570 nm. Each condition was determined in sextuplicate, and all results were repeated at least three times.

Flow cytometric analysis. DMSCs from different passages (3, 6 and 12) were seeded in six-well plates at the same density (3×10⁵ cells/well). After cell adherence for 24 h, the cells were washed with PBS twice and suspended in PBS (-20°C) containing 70% ethyl alcohol for 24 h. Subsequently, the cells were stained with propidium iodide (PI)/RNase staining solution in the dark for 30 min at 37°C, and analyzed using flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA).

Western blot analysis. The cells were lysed in RIPA buffer, Lysates were incubated for 30 min on ice and centrifuged at 13,201 × *g* for

Figure 1. Characterization of isolated dermal mesenchymal stem cells (DMSCs). A: Representative images from flow cytometry show the expression of surface markers of DMSCs isolated from newborn mice. Microscope images showing that isolated DMSCs can differentiate into adipocytes (B) and osteocytes (C). Scale bar: 100 μ m. FITC: Fluorescein isothiocyanate; PE: phycoerythrin.

Figure 2. Knockout of peroxiredoxin 2 (PRDX2) inhibits dermal mesenchymal stem cell (DMSC) growth. A-C: The growth curve of DMSCs from different passages (3 P, 6 P and 12 P). D: Western blot analysis showing that the proliferation-related signal in *Prdx2*^{+/+} (WT) and *Prdx2*^{-/-} (-/-) DMSCs is inhibited at different passages. E and F: Quantification of western blot analysis by ImageJ software. Data are presented as the mean $\pm$ SEM. Significantly different at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. GSK3 β : Glycogen synthase kinase 3 beta; PCNA: proliferating cell nuclear antigen; STAT3: signal transducer and activator of transcription 3.

Figure 3. Deletion of peroxiredoxin 2 (Prdx2) increases cell-cycle arrest of dermal mesenchymal stem cells (DMSCs) in G₀/G₁. A: Flow cytometric analysis showing cell cycle of DMSCs at three distinct passages. B: Quantification of G₀/G₁ phase at three distinct passages. Deletion of Prdx2 (Prdx2^{-/-}) led to G₀/G₁ cell-cycle arrest of DMSCs. C: Western blotting was used to measure the expression of cell cycle-associated proteins. D: Quantification of western blot analysis by ImageJ software. Significantly different at * $p < 0.05$, and *** $p < 0.001$. WT: Wild-type Prdx2^{+/+}.

Figure 4. Up-regulation of glycogen synthase kinase 3 beta (GSK3 β)/ β -catenin signaling in dermal mesenchymal stem cells (DMSCs) with knockout of peroxiredoxin 2 (Prdx2 $^{-/-}$). The expression of GSK/ β -catenin signaling proteins analyzed with immunofluorescence (A) and western blot (B-D) implied that lack of Prdx2 $^{-/-}$ inhibits DMSCs growth via GSK/ β -catenin signaling. *Significantly different at $p < 0.05$. WT: Wild-type Prdx2 $^{+/+}$.

5 min at 4°C. Proteins were boiled for 5 min and separated on a 12% polyacrylamide gel. Protein expression and phosphorylation were monitored with specific antibodies and chemiluminescent horseradish peroxidase substrate (ZSGB-BIO, Beijing, PR China). Primary antibodies used in this study were as follows: anti-PRDX2 (Abfrontier, Seoul, Republic of Korea), anti-proliferating cell nuclear antigen (PCNA), anti-signal transducer and activator of transcription 3 (STAT3), anti-p-STAT3, anti-p21, anti-p16, anti-cyclin D1, anti-AKT serine/threonine kinase 1 (AKT), anti-p-AKT, anti-GSK3 β , anti-p-GSK3 β , anti- β -catenin, anti-p- β -catenin and anti- α -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies used were, Goat anti-mouse and Goat anti-rabbit (ZSGB-BIO) and the images were quantified using Image J software (<https://imagej.nih.gov/ij/index.html>, National Institutes of Health, Bethesda, MD, USA).

Cellular immunofluorescence staining. DMSCs were cultured in 24-well plates at a density of 1.5×10^4 cells/well. After 24 h, cells were treated with 4% paraformaldehyde at room temperature to maintain cellular shape for 10 min and washed twice with PBS. After that, cells were incubated with β -catenin antibody (Beyotime, Haimen, PR China) for overnight. The cells were then treated with Alexa Fluor™ 633 dye (Invitrogen, Carlsbad, CA, USA) for 1 h and the

nuclei were labeled with Hoechst 33342 (Invitrogen) and finally positive cells were detected by fluorescence microscopy.

Statistical analysis. All of the data were analyzed by Student *t*-test. All results are expressed as mean $\pm$ SEM from at least three independent repeated experiments. Statistical significance was assumed for $p < 0.05$.

Results

Isolation and characterization of primary DMSCs. The DMSCs were isolated through the protocol described in the Materials and Methods, and then characterized by staining for CD106, CD44 and negative marker of CD14, CD34 and CD45 (15-18). As shown in Figure 1A, the isolated cells strongly stained with antibodies to CD106 and CD44, and low binding affinity with CD14, CD34 and CD45 antibodies. Since DMSCs have stem cell characteristics, we also examined the differentiation potential of the DMSCs. The results show that the isolated cells were strongly stained by red oil red O and alizarin red (Figure 1 B and C), suggesting

that the isolated DMSCs maintained stem cell characteristics, and were suitable for use in subsequent experiments.

Knockout of PRDX2 inhibits DMSC cell growth. To understand the effect of Prdx2 deletion on DMSC proliferation, the wild-type and Prdx2 knockout DMSCs were cultured for 1, 3, 5 and 7 days. The results showed that in early passages (passage 3), there were no significant differences between the growth of wild-type and Prdx2^{-/-} DMSCs (Figure 2A), while in the late passages (6 and 12), Prdx2^{-/-} DMSCs exhibited delay cell growth (Figure 2B and C) as detected by MTT assay. Therefore, we also checked the expression of PCNA and STAT3 proteins which are involved in cell proliferation. As shown in Figure 2D-F, with increasing culture passage, expression of PCNA protein was down-regulated in primary Prdx2^{-/-} DMSCs as was that of p-STAT3 protein.

Deletion of Prdx2 increases G₀/G₁ cell-cycle arrest of DMSCs. Since Prdx2 deletion inhibited DMSC growth (Figure 2), we hypothesized that it may affect the cell cycle processing, which is a key point of regulation of cell proliferation. To verify this, wild-type and Prdx2 knockout primary DMSCs were stained with PI/RNase solution to examine the cell cycle. The results showed that late passage (6 and 12 passage) DMSCs exhibited significant cell-cycle arrest, marked by G₀/G₁ cell accumulation, but not in early passages (passage 3) (Figure 3A and B). This strongly supports the cell growth results shown in Figure 2. In order to understand the possible molecular regulatory mechanism of PRDX2 in cell-cycle arrest, we also compared the expression of cell cycle-related proteins between wild-type and Prdx2^{-/-} DMSCs. The results showed that expression of p21 and p16 proteins, known as cell-cycle inhibitors, were significantly increased in Prdx2^{-/-} DMSCs, as well as cyclin D1 (Figure 3C and D).

Up-regulation of GSK/ β -catenin signaling in Prdx2^{-/-} knockout DMSCs. GSK3/ β -catenin is involved in various aspects of cell growth and differentiations through regulating the cell cycle related protein expressions (19-22). Our findings suggest that PRDX2 gene knockout could dramatically inhibit the cell growth by affect the cell cycle related proteins expressions (Figure 3), we hypothesises that PRDX2 knockout may also affect the GSK/ β -catenin signaling pathway. To verify this, we examined the β -catenin expression levels in the wild-type and Prdx2^{-/-} DMSCs. The result showed that up-regulation of β -catenin in Prdx2^{-/-} DMSCs compared with wild-type, as observed from immunocytochemistry (Figure 4A). Translocation of β -catenin is a mark of β -catenin activation, thus, we examined nuclear β -catenin protein expression between wild-type and Prdx2^{-/-} DMSCs. The results showed that deletion of Prdx2

increased the expression of nuclear β -catenin compared with wild-type DMSCs, while there was no significant difference in total cell lysate (Figure 4B). Since β -catenin is activated by GSK phosphorylation, we also assessed p-GSK and p-AKT expression between wild-type and Prdx2^{-/-} DMSCs. The results showed significant up-regulation of p-GSK and p-AKT in Prdx2^{-/-} DMSCs compared with wild-type (Figure 4C and D).

Discussion

DMSCs are pluripotent stem cells in the dermis (23-25). DMSCs can activate fibroblasts, stimulate secretion of collagen and promote the proliferation of fibroblasts and epidermal cells by secreting a series of cytokines such as tumor growth factor β , vascular endothelial growth factor, platelet-derived growth factor and hepatocyte growth factor (26, 27), therefore, the number and function of DMSCs play an important role in skin maintenance and homeostasis (28). DMSCs are a kind of seed cells suitable for tissue engineering because of their rapid proliferation, multi-directional differentiation and low immunogenicity (29). However, *in vitro* culture and proliferation of DMSCs is difficult, and it is easy to cause the loss of stemness, which limits its wide application. Therefore, there is a need to establish optimal culture conditions to study the key physiological and biochemical factors of DMSC self-renewal, so that DMSCs can maintain stemness and normal physiological functions after massive expansion to better research findings apply to clinical practice.

PRDX2 plays an important role in the proliferation and differentiation of many kinds of stem cells. Nitrosylation of PRDX2 can promote cardiac formation of mouse embryonic stem cells through X-box binding protein-1s/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway (30); PRDX2 has also been shown to regulate the differentiation of embryonic stem cells into neurons (31). Compared with primary stem cells, the regulation of stem cell stemness by PRDX2 comes more from the study of cancer stem cells (6, 32). Silencing PRDX2 gene resulted in the decrease of NANOG expression in colon cancer stem cells, and cell proliferation and migration were significantly reduced (6). The expression of SRY-related high-mobility-group-box protein-2 and octamer-binding transcription factor-4 was down-regulated in PRDX2-silenced Huh7-H-Ras^{G12V} hepatoma cells, and sphere formation efficiency and epithelial-mesenchymal transition were significantly inhibited (6). Mechanistic studies showed that PRDX2 can regulate stemness of cancer stem cells through a variety of signaling pathways, which contribute to the maintenance of the cancer stem cell properties of hepatocellular carcinoma *via* VEGF/EGFR/STAT3 signaling and RAS/FOXM1 signaling (33-35). In the process of isolation and expansion of DMSCs,

we found that knockout of PRDX2 can significantly inhibit the proliferation of stem cells with the increasing cell passage. *Prdx2*^{-/-} DMSCs exhibited cell-cycle arrest in the sixth and 12th passage; p16, p21 and cyclin D1 expression levels in *Prdx2*^{-/-} DMSCs were also significantly increased, indicating that knockout of the PRDX2 in mesenchymal stem cells leads to cell-cycle arrest, thereby affecting the proliferation of stem cells.

β -Catenin is a key element in driving the Wnt/ β -catenin signaling pathway, and GSK3 β is a critical kinase regulating β -catenin phosphorylation (36, 37). When the activity of GSK3 β kinase is inhibited, unphosphorylated β -catenin can accumulate in the cytoplasm without being degraded by proteasomes, and be transferred into the nucleus, where it binds to transcription factors and forms transcriptionally active complexes that initiate transcription of genes such as c-MYC, cyclin D1, and CD44(38), thereby regulating cell proliferation and differentiation (39). Our results showed that the expression of p-GSK3 β (Ser9) increased in *Prdx2*^{-/-} DMSCs with increasing cell passage, and β -catenin accumulated in the nucleus, indicating that β -catenin in the cytoplasm could not be phosphorylated and degraded by ubiquitination. The results of this study show that knockout of *Prdx2* in DMSCs can reduce the activity of GSK3 β and promote the translocation of β -catenin into the nucleus, thereby activating the downstream signaling pathways. However, activation of this signaling pathway did not improve cell proliferation caused by *PRDX2* knockout, high expression of cyclin D1 also did not prevent cell-cycle arrest (40). The kinase activity of GSK3 β can be regulated by different signaling pathways, in which phosphorylated AKT can phosphorylate GSK3 β to inactivate it (12, 37, 41). In *Prdx2*^{-/-} DMSCs, the phosphorylation of AKT increased as the number of cell passages increased, this is most likely achieved through cross talk between the PRDX2, PDGF and PI3K signals (42).

We conclude that PRDX2 plays an important role in regulating the proliferation of DMSCs, and this regulation is closely related to the AKT/GSK3 β / β -catenin signaling pathway. However, the specific regulatory mechanism involved remains to be further studied.

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Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Authors' Contributions

Y.H.H., M.H.J., T.K. and H.N.S. performed the experiments and wrote the article. Y.H.J., N.N.Y., J.L., Y.Q.Z., Y.D.C., A.G.W., D.S.L., S.U.K., J.S.K. and Y.H.H. performed the data analysis. Y.H.H., T.K., H.N.S. made substantial contributions to conception and design. All Authors read and approved the final article.

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