PARP1 Regulates Cellular Processes Mediated by Exosomal miRNAs in Dental Pulp Stem Cells

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Abstract: Non-coding RNAs including microRNAs (miRNAs) derived from extracellular exosomes are considered as biomarkers for multiple intracellular communication pathways. Meanwhile, poly ADP-ribosylation (PARylation) catalyzed by poly (ADP-ribose) polymerase 1 (PARP1) is related to various intracellular processes. The comprehensive idea of cell-cell signaling phenomena mediated by PARP1-related exosomes remains unsolved, although individual molecules are considered to provoke disease pathogenesis and progression under aberrant regulation. In the present study, we knocked down the PARP1 gene in dental pulp stem cells (DPSCs) by gene targeting using the CRISPR-Cas9 system to determine the functions of exosomal miRNAs regulated by PARP1. The exosomes produced by PARP1-knockdown DPSCs were harvested and the miRNAs contained within these exosomes were comprehensively analyzed by next-generation sequencing. From the results, significantly altered miRNAs were picked up among the detected miRNAs. Gene ontology enrichment analyses were performed on these miRNAs to predict their cellular functions. Most of the up-regulated miRNAs after PARP1 knockdown were identified as cell proliferation-related functional non-coding RNAs, and were indicated to affect cellular processes regulating cellular senescence and differentiation. Therefore, the present findings suggest that PARP1 in DPSCs regulates cellular processes such as cell proliferation through intercellular communication mediated by exosomal miRNAs.

Key words: Dental pulp stem cell (DPSC), Exosome, microRNA (miRNA), Poly (ADP-ribose) polymerase 1 (PARP1)

Introduction

In 1981, the first report of exosomes, defined as small membrane-bound vesicles secreted by exocytosis, was published by Trams et al.1 Exosomes include informative components like proteins, messenger RNAs (mRNAs), and microRNAs (miRNAs) and participate in intercellular communication pathways.2,3 Secreted exosomes stimulate surrounding cells in many situations, and are consequently involved in extensive biological functions. Numerous studies have clarified that exosomes are produced by various types of cells, including hematopoietic cells, cardiomyocytes, vascular endothelial cells, and fibroblasts.4,5 Bruno and colleagues revealed that exosomes produced by mesenchymal stromal cells (MSCs) promote wound healing by stimulating cells adjacent to the wounded tissue.6 Exosomes are also implicated in a variety of diseases. Moreover, exosomes produced by tumor cells provoke malignant progression with invasion, acquisition of drug resistance, and immune system evasion by tumor cells.7-10 Dental pulp stem cells (DPSCs), established from extracted tooth pulp, exhibit high proliferation activity and have the potential to differentiate into bone, cartilage, and adipose cells, similar to the case for MSCs9,10. DPSCs are also suggested to produce many exosomes, and these exosomes are considered to promote DPSC proliferation and differentiation.11-14

The post-translational protein modification known as poly ADP-ribosylation (PARylation) is catalyzed by poly (ADP-ribose) polymerase member 1 (PARP1).15 PARP1 is a 113-kDa nuclear enzyme that behaves as a protective molecule in DNA damage responses and in various cellular processes such as differentiation, proliferation, and transformation of tumor cells.16-18. A previous study indicated that PARP inhibitors down-regulated the osteogenic differentiation potential of mouse MSCs.19 Therefore, PARP1 is thought to be involved in the regulation of stemness in somatic stem cells. From another point of view, analysis of miRNAs in Parp1-knockout mouse embryonic stem (ES) cell-derived exosomes showed that Parp1 affected intercellular communication through miRNAs contained within the exosomes.20 Nevertheless, the functional significance of PARP1 for exosome secretion and the role of exosomes modulated by PARP1 in DPSCs remain uncertain. In the present study, we performed a comparative analysis of the expressions of exosomal miRNAs by next-generation sequencing (NGS) in PARP1 gene-targeted human DPSCs and control DPSCs to determine the functions of exosomal miRNAs regulated by PARP1.

Materials and Methods

Preparation of human DPSCs

Human DPSCs were purchased from Lonza Group AG (Lonza, Basel, Switzerland). These cells were isolated from adult third molars collected during extraction of wisdom teeth. The cells were cultured in DPSC basal medium (Lonza) containing DPSC SingleQuots (Lonza) at 37°C under 5% CO2 in air.
Isolation of exosomes and NGS analysis of small RNAs were performed as previously described \(^19\). Briefly, the medium supernatant of cultured cells was collected by centrifugation at 2000×g for 30 min at 4°C, and filtered through a Millex-GV 0.22-μm filter (Merck Millipore Ltd., Burlington, MA, USA). Exosomes were isolated from the supernatant of cultured cells by centrifugation at 100,000×g for 70 min at 4°C, and filtered through a 0.22-μm filter (Merck Millipore Ltd., Burlington, MA, USA). Exosomes were isolated from the supernatant using the exoRNeasy Serum/Plasma Maxi Kit (Qiagen Ltd., Venlo, Netherlands). Next, total RNAs were extracted by an acid-phenol method, and the quality of the purified RNAs was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Ltd., Santa Clara, CA, USA). Subsequently, miRNA libraries were prepared using a QIAseq miRNA Library Kit (Qiagen) according to the manufacturer’s instructions. NGS was performed with 50-bp single reads using a HiSeq 1500 platform (Illumina Inc., San Diego, CA, USA). The enriched regions were detected, and the types of small RNAs were classified. All procedures for exosome-derived small RNA preparation and NGS analysis were performed by DNA Chip Research Inc. (Tokyo, Japan).

Table 1. crRNAs used for PARP1 gene targeting in this study

| crRNA | Sequence | PAM sequence | Strand |
|-------|----------|--------------|--------|
| A     | 5'-GAGTCGAGTACGCCAAGGC-3' | GGG | – |
| B     | 5'-ATTGACCCGTGGTACCATCC-3' | AGG | – |
| C     | 5'-CTCAACGTCAGGGTGCAGGA-3' | TGG | + |
| D     | 5'-AAGTACGTCAGGAGGTGTA-3' | TGG | – |

PAM: proto-spacer adjacent motif.

**Gene targeting of the PARP1 gene using the Crisper/Cas9 system**

The PARP1 gene was targeted using the Alt-R CRISPR-Cas9 system (Integrated DNA Technologies Inc., Coralville, IA, USA). The CRISPR RNAs (crRNAs) used in this study are shown in Table 1. To construct the guide RNA (gRNA) complex, each crRNA was mixed with a trans-activating crRNA (tracrRNA), heated to 95°C for 5 minutes, and cooled down. The gRNA complex was then mixed with Cas9 Nuclease (TrueCut Cas9 Protein v2; Thermo Fisher Scientific Inc., Waltham, MA, USA) to construct the ribonucleoprotein (RNP) complex. The RNP complex and a puromycin-N-acetyltransferase expression vector, used for screening the complex and vector-transfected cells, were transfected at a complex-to-vector ratio of 1:10.1 using the Nucleofector system (Human MSC Nucleofector Kit and Nucleofector 2b Device; Lonza). The transfected cells were selected with puromycin for 7 days and cell clones were established. The knockdown efficiency was evaluated using a PARP/Apoptosis Assay Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Immunofluorescence staining**

DPSCs cultured on 24-well cell culture plates (BD Falcon Inc., Franklin Lakes, NJ, USA) were fixed with 4% paraformaldehyde, permeabilized with Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s Phosphate-Buffered Saline (PBS; Nacalai Tesque Inc., Kyoto, Japan) containing 0.1% Triton X-100, and blocked with PBS containing 5% chicken serum to reduce non-specific binding. The cells were then incubated overnight at 4°C in Saline (PBS; Nacalai Tesque Inc., Kyoto, Japan) containing 0.1% Triton X-100, and blocked with PBS containing 5% chicken serum to reduce non-specific binding. The cells were then incubated overnight at 4°C in a humidified chamber with an anti-PARP1 primary antibody (rabbit anti-human PARP1 antibody; 46D11; Cell Signaling Technology Inc., Danvers, MA, USA) at 1:400 dilution, followed by incubation with an Alexa Fluor 647-conjugated secondary antibody (Thermo Fisher Scientific) for 30 minutes at room temperature. Cellular filamentous actin was counterstained with Alexa Fluor 488-Phalloidin (Thermo Fisher Scientific), and the cells were observed with a BZ-9000 fluorescence microscope (Keyence Corp., Osaka, Japan).

**Cell cycle analysis**

Cell cycle analysis was performed using the Tali cell cycle reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. PARP1-knockdown DPSCs and control DPSCs were seeded onto 6-well cell culture plates (BD Falcon) and cultured for 2 days. Then, cells were harvested using accutase (Nacalai Tesque) and washed with PBS. Cells were fixed with ice-cold 70% ethanol overnight, at −20°C. Cells were stained with Tali cell cycle reagent and analyzed using the Tali image-based cytomter (Thermo Fisher Scientific). The proportion of cells at different cell cycle phases were counted and quantified based on the fluorescence intensity of propidium iodide.

**Isolation of exosomes and NGS analysis of small RNAs**

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**Morphological and cell cycle analysis of PARP1-targeted DPSCs**

Immunofluorescence analysis was performed to confirm the PARP1 knockdown efficiency. As shown in Fig. 2A, a clone of PARP1-knock-
Figure 2. PARP1 expression and cell cycle arrest of PARP1-knockdown DPSCs. (A) Immunofluorescence staining of PARP1-knockdown DPSCs. Representative fluorescence images of filamentous actin with phalloidin staining (upper panels: green) and PARP1 (lower panels: red) are shown. PARP1 expression is recognized in control DPSCs (left panel), but is not detected in PARP1-knockdown DPSCs with crRNA (A) (right panel). Scale bars: 100 μm. (B) Representative phase-contrast images of (a) control DPSCs and (b, c) PARP1-knockdown DPSCs. Scale bars: 100 μm. (C) The cell cycle analysis of control DPSCs (gray bars) and PARP1-knockdown DPSCs (open bars). These values were compared between control and knockdown cells: bars represent the mean ± S.D. of three independent measurements. The data were evaluated by student t-tests with Sidak-Bonferroni’s comparison procedure. Statistical significance was defined as the following p-values: * P < 0.01, N.S.: not significant.
down DPSCs with crRNA (A) lacked PARP1 expression. Therefore, this DPSC clone was considered to exhibit knockdown of the PARP1 gene. We finally established three subclones from the PARP1-knockdown DPSCs with crRNA (A). However, two of the subclones showed the cell morphology of senescence: flat and larger morphologies, and decreased cell growth during the early stages of culture (Fig. 2B-b). The remaining PARP1-knockdown clone did not show severe morphological defects in early passages (Fig. 2B-c). However, this clone indicated the higher cell cycle arrest in G2/M phase (Fig. 2C). Therefore, the remaining clone of PARP1-knockdown DPSCs with crRNA (A) was used for subsequent experiments.

**Profiling of miRNA expressions in PARP1-knockdown and control DPSCs**

PARP1-knockdown DPSCs and control DPSCs were cultured on a large scale, and the exosomes extracted from their culture supernatants were subjected to NGS analysis. Based on the NGS analysis, the numbers of up-regulated and down-regulated miRNAs with ≥2-fold expression changes under PARP1 deficiency were 163 and 171, respectively (Fig. 3A). From these up-regulated miRNAs with ≥2-fold expression changes, we focused on the miRNAs showing ≥2-fold changes between control DPSCs and PARP1-knockdown DPSCs and having read counts >150 in PARP1-knockdown DPSCs are indicated. The read counts indicated on the Y-axis are the raw data values.

![Figure 3. Profiling of exosomal miRNA expressions. (A) Dot blot analysis for the expression levels of exosomal miRNAs. The read counts of all detected miRNAs were normalized and plotted. Data outside the gray lines represent miRNAs with ≥2-fold changes in expression. The dotted line indicates the linear regression. (B) Comparison of miRNA read counts. The miRNAs showing ≥2-fold changes between control DPSCs and PARP1-knockdown DPSCs and having read counts >150 in PARP1-knockdown DPSCs are indicated. The read counts indicated on the Y-axis are the raw data values.](image-url)
changes, ten miRNAs with high read counts were obtained (Fig. 3B).

**GO enrichment analysis for predicted functions of exosomal miRNAs**

To predict the functions of the miRNAs produced by PARP1-knockout DPSCs, GO enrichment analysis was performed. The results revealed some characteristic GO terms related to cell cycle regulations, transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling pathways, differentiation potentials, chromatin modifications, and transcription activities (Fig. 4). These results suggested that the exosomal miRNAs produced by PARP1-knockdown DPSCs affected various cellular processes such as cell growth.

**Discussion**

The contribution of PARylation regulated by PARP1 to cell-cell signaling has remained unclear, despite its involvement in various intracellular processes\(^\text{16,17}\). Meanwhile, exosomes secreted by cells are considered to act as intercellular communication molecules\(^\text{2}\). In fact, many studies have shown that the exosomes secreted by MSCs participate in regeneration processes after tissue injury through cell-cell communication\(^\text{16,17}\). DPSCs also produce exosomes and these exosomes are considered to

Figure 4. GO analysis for predicted functions of exosomal miRNAs. GO terms, defined by the GO Consortium, were extracted by GO enrichment analysis and classified. Corrected P-values (−log\(_10\)) were calculated from the P-values. Up-GO Term: GO terms increased by PARP1 knockdown; Down-GO Term: GO terms decreased by PARP1 knockdown.
promote DPSC proliferation and differentiation. PARP1 expression is up-regulated in DPSCs through oxidative stress caused by endodontic materials. Valverde and colleagues reported that PARP1 functions as a negative regulator for brain-derived neurotrophic factor secretion during odontogenic differentiation. However, the functional significance of PARP1 for exosome secretion and the role of the exosomes modulated by PARP1 in DPSCs remain unknown.

In the present study, we analyzed the exosomal miRNAs that exhibited marked changes after PARP1 knockdown. As shown in Fig. 3B, most of the up-regulated miRNAs were related to cell proliferation potential. In previous studies, certain miRNAs were shown to suppress the proliferation, migration, and invasion of various cell types (miR148a-3p: osteosarcoma cells; miR-99a-5p: oral carcinoma cells; miR-101-3p: salivary gland adenoid cystic carcinoma cells; miR-195-5p: nerve cells in Hirschsprung disease; miR-497-5p: non-small cell lung cancer cells) (20-23). Meanwhile, miR-424-5p was found to repress intrahepatic cholangiocarcinoma metastasis and invasion, while miR-224-5p was shown to suppress renal cell carcinoma cell proliferation and induced cell cycle arrest (24-25). In particular, miR-486-5p was reported to induce replicative senescence in human adipose tissue-derived mesenchymal stem cells (26). In our GO analysis, it was predicted that cellular processes such as cell cycle regulations and chromatin replication changes were affected by PARP1 knockdown (Fig. 4). Specifically, the results suggested that the miRNAs up-regulated by PARP1 knockdown suppressed cell proliferation activities. Indeed, the DPSCs showed decreased cell proliferation and cell cycle arrest in G2/M phase after PARP1 knockdown (Fig. 2B-b and C). Recent studies advocated that G2/M checkpoint are key factors for the onset of replicative senescence (21). Therefore, it is suggested that PARP1 in DPSCs inhibits the production of miRNAs that regulate cell proliferation through suppression.

In a previous study, TGF-β stimulation was suggested to promote cellular senescence of MSCs (27). Moreover, another study indicated that BMP signaling was involved in PARP1-mediated regulation of osteogenic differentiation (28). As shown in Fig. 4, changes in TGF-β and BMP signaling and differentiation potentials were predicted after PARP1 knockdown. From the above findings, it is suggested that PARP1 affects TGF-β and/or BMP signaling, and regulate cellular senescence and differentiation potential.

In conclusion, the present study demonstrated that PARP1 in DPSCs regulates cellular processes such as cell proliferation through intercellular communication mediated by exosomal miRNAs. The present findings suggest that rejuvenation or functional regeneration of DPSCs may be possible through regulation of PARP1.

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Conflict of Interest
The authors declare no competing financial interests.

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