Extracellular vesicles from deciduous pulp stem cells recover bone loss by regulating telomerase activity in an osteoporosis mouse model

CURRENT STATUS: UNDER REVIEW

Soichiro Sonoda
Kyushu University

Sara Murata
Kyushu University

Kento Nishida
Kyushu University

Hiroki Kato
Kyushu University

Norihisa Uehara
Kyushu University

Yukari N. Kyumoto
Kyushu University

Haruyoshi Yamaza
Kyushu University

Ichiro Takahashi
Kyushu University

Toshio Kukita
Kyushu University

Takayoshi Yamaza
Kyushu University

yamazata@dent.kyushu-u.ac.jp

Corresponding Author

ORCiD: https://orcid.org/0000-0001-7055-3370

DOI:
SUBJECT AREAS

Stem Cell & Developmental Cell Biology  Orthopedics

KEYWORDS

Human deciduous pulp stem cells, Extracellular vesicles, MicroRNA, Telomerase activity, Osteoporosis
Abstract
Background: Systemic transplantation of stem cells from human exfoliated deciduous teeth (SHED) induces bone regeneration in animal models of osteoporosis; however, the mechanisms underlying this remain unclear. Here, we hypothesized that trophic factors within SHED-releasing extracellular vesicles (SHED-EVs) rescue osteoporotic phenotype.

Methods: EVs were isolated from culture supernatant of SHED. SHED-EVs were treated with or without ribonuclease and systemically administrated into ovariectomized mice, followed by the measurement of bone regenerative function of recipient bone marrow mesenchymal stem cells (BMMSCs) including Tert expression. Subsequently, human BMMSCs were stimulated by SHED-EVs with or without ribonuclease treatment, and then human BMMSCs were examined osteogenic function in vitro and telomerase activity. Furthermore, SHED-EVs treated human BMMSCs were subcutaneously transplanted into dorsal skin of immunocompromised mice with hydroxyapatite tricalcium phosphate (HA/TCP) careers and analyzed the de novo bone forming ability.

Results: We revealed that systemic SHED-EV-infusion recovered bone volume in ovariectomized mice and improved osteogenic function of recipient BMMSCs by rescuing the mRNA levels of Tert and telomerase activity. Ribonuclease treatment depleted RNAs, including microRNAs, within SHED-EVs and these RNA-depleted SHED-EVs attenuated SHED-EV-triggered bone regeneration and telomerase-mediated osteogenesis in the ovariectomized mice. These findings were supported by in vitro osteogenic assays using human BMMSCs incubated with SHED-EVs.

Conclusion: Collectively, our findings suggest that SHED-secreted trophic factors, specifically microRNAs, play a crucial role in treating postmenopausal osteoporosis by targeting the telomerase activity of recipient BMMSCs.

Background
Stem cells from human exfoliated deciduous teeth (SHED), isolated from the pulp tissues of deciduous teeth, are mesenchymal stem cells (MSCs) that exhibit self-renewal properties and differentiate into osteoblasts, chondrocytes, adipocytes, neural cells, endothelial cells, and hepatocytes [1, 2]. SHED also exhibit improved colony formation, proliferation, and immunomodulation properties and are less
tumorigenic than bone marrow MSCs (BMMSCs) [3, 4]. Human clinical trials aimed at treating trauma-induced tooth injury [5] as well as animal pre-clinical studies for diseases including systemic lupus erythematosus, spinal injury, and Wilson’s disease [2, 3, 6, 7] have reported SHED to be promising candidates for cell-based therapies [8]. For instance, systemic SHED transplantation-based therapy employs multiple mechanisms, including direct conversion into target tissue-specific cells, release of trophic factors, and cell-cell contact [3, 6, 7, 9-11].

Recent studies have also shown that systemic SHED transplantation increases bone volume in a Fas-mutated MRL/lpr mice model for systemic lupus erythematosus and ovariectomy-induced (OVX) mice model for postmenopausal osteoporosis [12, 13]. Osteoporosis hinders the functions of BMMSCs [14, 15]. Specifically, systemic SHED transplantation was found to rescue BMMSC function in recipient MRL/lpr and OVX mice [12, 13]. Further, the interplay between SHED and other immune cells, especially T cells, in immunomodulation via the Fas pathway improves symptoms of osteoporosis, with low numbers of engrafted SHED observed in recipient bone tissues, suggesting that systemic SHED transplantation regulates cells to rescue recipient BMMSC function [13]. However, the mechanism(s) involved in this process remains uncharacterized.

Telomerase reverse transcriptase (TERT) is the main catalytic telomerase subunit that is required to maintain telomere length [16]. Although telomerase activity is undetectable in most normal human somatic cells, it is exhibited to varying degrees in stem/progenitor cells [17], which induces feedback regulation [18]. This activity confers properties of self-renewal, proliferation, osteogenic differentiation, and tissue regeneration in MSCs [19-21] and is also involved in immunomodulating donor MSCs to improve systemic sclerosis-like symptoms in Tsk/+ mice [22]. However, whether telomerase activity can be targeted by SHED-based therapy in recipient organs/tissues/cells, has to be elucidated.

Extracellular vesicles (EVs) are membrane-bound vesicles secreted from cells that exist as either exosomes (40–100 nm in diameter) or plasma membrane-derived microvesicles (100–1000 nm in diameter). Stem cell-derived EVs contain enriched small RNAs, mostly microRNAs (miRNAs) as compared to parental cells, which mediate cell-cell communication [23]. SHED release EVs, including
exosomes and microvesicles [24], that contain various bioactive molecules, such as proteins and RNAs, which participate in intercellular communication and therapeutic effects [25]. Administration of SHED-released EVs (SHED-EVs) abrogate symptoms associated with acute inflammation, brain injury, and Parkinson’s disease [26–28]. However, the mechanisms involved in the therapeutic and cellular properties of SHED-EVs remain to be examined in the context of postmenopausal osteoporosis and recipient BMMSCs. Thus, in the current study, we identified important roles of SHED-EVs in rescuing the recipient BMMSC function via activation of telomerase activity, which may suggest a novel therapeutic strategy for postmenopausal osteoporosis.

Methods

Mice

C57BL/6J mice (female, 8 weeks old) and Balb/c nu/nu mice (female, 10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyushu University (Approval Number: A21-044-1).

Antibodies

Additional file 1: Supplementary Table1 lists the antibodies used in this study.

Isolation, culture, and characterization of SHED, human BMMSCs, and mouse BMMSCs

SHED, human BMMSCs (hBMMSCs), and mouse BMMSCs (mBMMSCs) were isolated and cultured according to previous reports [1,29]. The isolated cells were characterized as MSCs according to the published criteria [30]. The details have been described in the Additional file 1: Supplementary Methods.

Isolation, characterization, and ribonuclease A treatment of SHED-EVs

Conditioned medium (CM) was collected from three-day SHED cultures with Dulbecco’s minimum essential medium low glucose type (Thermo Fisher Scientific, Waltham, MA). The CM was centrifuged at 500 xg for 5 min and used for SHED-EV isolation using the exoEasy Maxi kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. SHED-EV particle size was measured using the qNano analyzer (Izon Science, Christchurch, New Zealand). A fraction of the SHED-EVs were treated with ribonuclease A (RNase A; 5 U/mL; Thermo Fisher Scientific) at 37 °C for 3 h and incubated with RNase
inhibitor (40 U/mL; Thermo Fisher Scientific) at room temperature for 10 min followed by ultracentrifugation at 110,000 × g for 1 h. SHED-EVs were subjected to Flow cytometry (FCM) analysis using the ExoAB Antibody kit (Cat. No. ExoAB-KIT-1, System Bioscience, Palo Alto, CA) and R-phycoerythrin-conjugated anti-rabbit IgG (Cat. No. 8885s, Cell Signaling Technology, Danvers, MA) according to the manufacturer instructions. Total proteins were extracted from the SHED-EVs and SHED using the M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with proteinase inhibitor cocktail (Nacalai Tesque) and quantified using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) following which they were used for western blotting. Total RNA was extracted from SHED-EVs using a miRNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer (Agilent Santa Clara, CA).

Systemic infusion of SHED and SHED-EVs into mice with postnatal osteoporosis

Ovariectomized female C57BL/6J mice (10 weeks old; OVX mice) were intravenously administered SHED (0.1 × 10⁶/100 mL of phosphate-buffered saline (PBS)/10 g body weight) and SHED-EVs (100 μg in 100 μL PBS) pretreated with or without ribonuclease A (RNase) two days post-surgery and sacrificed four weeks post-surgery. Age-matched sham-operated C57BL/6J and OVX mice infused with PBS (100 mL/10g body weight) served as experimental controls.

Colony forming units-fibroblast assays

Colony forming units-fibroblast (CFU-F) assays was performed as described in the Additional file 1: Supplementary Methods.

Cell proliferation assay

Bromodeoxyuridine (BrdU)-uptake assay was performed as described in the Additional file 1: Supplementary Methods.

Surface antigen analysis

BMMSCs (0.1 × 10⁶) were stained with R-phycoerythrin-conjugated antibodies (1 μg) or isotype-matched control antibodies and analyzed by FCM as described in the Additional file 1: Supplementary Methods.
**In vitro osteogenesis**

Osteogenesis by mouse and human BMMSCs were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Alizarin Red-S staining according to previous studies [29] as described in the **Additional file 1: Supplementary Methods**.

**In vivo osteogenesis**

hBMMSCs (4.0 × 10^6) were mixed with hydroxyapatite tricalcium phosphate (HA/TCP) particles (40 mg, Zimmer, Warsaw, IN) and subcutaneously implanted under the dorsal skin of immunodeficient Balb/c nu/nu mice (female, 10 weeks old) as described in the **Additional file 1: Supplementary Methods**.

**Telomerase activity analysis**

Telomerase activity was analyzed by quantitative telomerase repeated amplification protocol (RQ-TRAP) using a quantitative telomerase detection kit (Cat. No. MT3010, Allied Biotech, Inc., Ijamsville, MD) according to the manufacturer’s instructions as reported previously [29]. HEK293T cells were used as the positive control. Heat-inactivated cell lysates were used as negative controls.

**Bone analysis by micro-computed tomography**

BMD and bone structural indices: trabecular bone volume versus total volume (BV/TV), trabecular numbers (Tb.N), and trabecular thickness (Tb.Th) of mouse third lumbar vertebrae (L3) were analyzed using a 1076 micro-computed tomography (micro-CT) system micro-CT scanner (Skyscan, Kontich, Belgium) and the CTAn software (Skyscan) as described previously [12]. Density values were calibrated using hydroxylapatite phantoms with BMD values of 0.25 and 0.75 g/cm^3 (Skyscan).

**In vivo and in vitro tracing assays**

Carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific) or PBS was used as labels according to the kit instructions. CSFE-SHED (0.1 × 10^6/100 mL of PBS/10 g body weight) were intravenously infused into OVX mice (10 weeks old) 2 days post-surgery. After 7 days of infusion, prepared frozen sections were mounted using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Cat. No. H-1200-10, Vector Laboratories, Burlingame, CA, USA). CSFE-
labeled SHED-EVs (20 mg/mL) were incubated with cultured hBMMSCs for 3 days and subjected to histological and FCM analyses.

*In vivo osteoclast activity analysis*

Paraffin sections of bone samples were treated with tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells were analyzed as described previously [29]. Serum C-terminal telopeptides of type I collagen (CTX) and soluble receptor activator for nuclear factor κB ligand (sRANKL) were measured by the RatLaps enzyme-linked immunosorbent assay (ELISA) (Cat. No. DS-AC06F1, Nordic Bioscience Diagnostics A/S, Herlev, Denmark) and Mouse TRANCE/RANKL/TNFSF11 Quantikine ELISA kits (Cat. No. MTR00, R&D Systems, Minneapolis, MN), respectively.

*In vitro osteoclast activity analysis*

Mouse bone marrow cells (BMCs; 1 × 10^6 cells/well) were co-cultured with mouse newborn calvarial osteoblastic cells (1 × 10^5 cells/well) for 7 days as per a published protocol [29] as described in the **Additional file 1: Supplementary Methods**. The number of TRAP-positive multinucleated cells (> 3 nuclei) and expression of osteoclast markers were analyzed.

**Statistical analyses**

The data were expressed as mean ± standard error of the mean or mean ± standard deviation from triplicates. Comparisons between two groups were analyzed by an independent two-tailed Student’s t-test. Multiple group comparisons were performed by a one-way analysis of variance followed by Tukey’s post hoc test. Kaplan-Meier and Kruskal-Wallis test were used for the survival assays. P < 0.05 were considered statistically significant. All statistical analyses were performed using the PRISM 6 software (GraphPad, Software, La Jolla, CA, USA).

**Results**

**Systemic SHED transplantation rescues impaired BMMSC function through telomerase activity in recipient OVX mice**

In this study, female C57BL/6 mice (10 weeks old) were ovariectomized and used to understand whether estrogen deficiency affects BMMSCs. The CFU-F and BrdU assays showed enhanced colony formation and decreased proliferation, respectively, in BMMSCs derived from OVX mice (OVX-
BMMSCs) in comparison to that of BMMSCs derived from Sham-mice (Figures 1a, 1b). FCM results revealed that only CD146 and CD73 levels were decreased in OVX-BMMSCs compared to Sham-BMMSCs (Figure 1c). Furthermore, Alizarin Red staining and RT-qPCR revealed that OVX-BMMSCs exhibited impaired osteogenesis in osteogenic inductive conditions as observed by a reduction in formation of mineralized nodules and mRNA levels for osteogenic markers, including Runt-related transcription factor 2 (Runx2) and bone gamma carboxyglutamic acid protein (Bglap) (Figures 1d-1f). OVX-BMMSCs also showed decreased expression of the osteoprotective factor Semaphorin-3a (Sema3a) under these conditions (Figure 1g). Meanwhile, results from CFU-F, BrdU, and FCM assays showed that SHED transplantation rescued the abnormally functioning recipient BMMSCs (SHED transplanted mice derived BMMSCs: SHED-BMMSCs) as seen by decreased colony formation as well as increased cell proliferation, and CD146 and CD73 levels. We also detected increased mineralized nodule formation and mRNA levels of Runx2, Bglap, and Sema3a under osteogenic conditions, indicating recovery of the impaired osteogenic function of recipient SHED-BMMSCs (Figures 1a-1g). Thus, SHED transplantation appears to rescue the impaired function of recipient BMMSCs and bone reduction in OVX mice.

Since low telomerase levels have been shown to regulate osteogenesis by BMMSCs [19,22], we next examined the difference in telomerase activity between OVX-BMMSCs and Sham-BMMSCs. OVX-BMMSCs showed a significant decrease in telomerase activity in comparison with that of Sham-BMMSCs as determined via the real-time RQ-TRAP (Figure 1h). Interestingly, SHED transplantation recovered the telomerase activity of recipient BMMSCs in OVX mice (Figure 1h).

Additionally, TERT has been shown to play an essential role in regulating telomerase activity [16]. We, therefore examined whether TERT contributes to the improved telomerase activity in recipient OVX-BMMSCs, and confirmed, via RT-qPCR, rescue of the Tert mRNA level in OVX-BMMSCs upon SHED transplantation (Figure 1i). Further, although SHED were detected in the bone marrow seven days after the infusion; CFSE labeling showed no significant difference in the number of SHED in the recipient bone and bone marrow (Figure 1j). Hence, these results indicate that the status of TERT-associated telomerase activity is crucial for the SHED transplantation-mediated rescue of impaired
recipient BMMSC functioning and bone reduction in OVX mice.

Systemic SHED transplantation recovers bone loss in postmenopausal OVX mice

Postmenopausal osteoporosis is a common systemic skeletal disease in elderly women that results from an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts, thereby leading to reduced BMD and deteriorated bone microarchitecture that is a high risk factor for fragility fractures [31]. Systemic SHED transplantation has been found to ameliorate bone reduction in an ovariectomized mouse model for postmenopausal osteoporosis [12,15]. Hence, in the current study, OVX mice received SHED (0.1×10^6/10 g body weight) two days post-surgery and were used to determine the osteoporotic phenotype of the L3 four weeks post-transplantation. Increased BMD, BV/TV, Tb.N, and Tb.Th were observed by micro-CT, indicating rescue of the osteoporotic phenotype in the treated OVX mice as compared to the control mice (Additional file 1: Supplementary Figure 1).

Enhanced in vivo osteoclast activity in OVX mice was rescued four weeks after SHED transplantation was performed, as evidenced by the observed decrease osteoclast number as well as serum levels of the sRANKL and CTX following TRAP staining and ELISA (Additional file 1: Supplementary Figure 2). Further, the in vitro osteoclast activity of OVX mice was rescued four weeks after SHED transplantation as indicated by the decrease in TRAP-positive multinuclear cells (MNCs) and mRNA levels of the osteoclast markers, including receptor activator for nuclear factor κB (Rank), nuclear factor of activated T-cell (Nfatc1), and cathepsin K (Ctsk) by TRAP staining and RT-qPCR following coculturing of calvarial osteoblasts from wild-type newborn C57BL/6 mice stimulated with prostaglandin E2 (Additional file 1: Supplementary Figure 3). These results suggest that systemic SHED infusion rescues osteoporotic phenotypes and improves the differentiation and function of osteoclast-lineage cells.

Infusion of SHED-secreted EVs ameliorate bone reduction in OVX mice

Since SHED transplantation increased Tert mRNA levels to rescue telomerase activity in recipient BMMSCs, we hypothesized that trophic factors released from SHED contribute to the SHED transplantation-mediated rescue of impaired Tert mRNA expression, telomerase activity, and bone
loss in OVX mice. Particularly, trophic factors within MSC-secreting EVs mediate cell-cell communication to deliver extracellular signals, thereby leading to therapeutic effects [32,33]. Thus, we isolated EVs from the SHED-conditioned medium harvested from a 3-day culture and examined the characteristics of the SHED-EVs. Results show that SHED-EVs were 57–272 nm in diameter with a mean of 86±2.5 nm, as determined using particle tracking analysis (Figure 2a). The concentration of SHED-EVs in the CM was $1.6 \times 10^9 \pm 4.8 \times 10^8$ particles/mL. Moreover, SHED-EVs significantly expressed exosome markers, such as CD9 (83.3 ± 2.5%), CD63 (42.8 ± 3.3%), and CD81 (85.9 ± 6.2%), and reduced expression of the MSC surface marker CD90 (4.1 ± 1.8%) as detected by FCM (Figure 2b). Western blotting showed an enrichment of CD63 and CD81 in the SHED-EVs, however, the same was not observed for calnexin (an endoplasmic reticulum marker), as compared to that SHED (Figure 2c). We also detected miRNAs within SHED-EVs (the concentration of miRNAs and small RNAs were $2.8 \pm 0.46$ ng/mL and $4.6 \pm 0.83$ ng/mL, respectively; the proportion of miRNA content in the small RNA was 61.1 ± 2.4%; Figure 2d). The total protein content of SHED-EVs was 828.3 ± 65.8 mg/mL. Next, we depleted the small RNA content, especially miRNAs, of SHED-EVs by treating with RNase (5 U/mL) for 30 min at 37 °C (resulting concentrations of miRNAs and small RNAs were $1.5 \pm 0.43$ ng/mL and $2.3 \pm 0.28$ ng/mL, respectively; the proportion of miRNA content in the small RNA was 62.8 ± 3.8%; Figure 2e); RNase treatment did not affect the particle size of the SHED-EVs (Figure 2f) nor their membrane surface phenotype and total protein content (data not shown). These findings indicate that SHED-EVs released into the CM are miRNA-enriched exosomes. Next, to determine whether EVs released from SHED are an alternative to SHED themselves in postmenopausal osteoporosis therapy, we intravenously infused SHED-EVs (100 μg/mouse) into OVX mice two days post-surgery. After four weeks of intravenously administering SHED-EVs, the osteoporotic phenotype of OVX mice was observed to be rescued as indicated by the marked increase in BMD, BV/TV, Tb.N, and Tb.Th by micro-CT analysis (Figures 3a-3c). The enhanced in vivo and in vitro osteoclast activity in OVX mice was also rescued four weeks after administering SHED-EVs, as indicated by the decrease in the TRAP-positive cells and serum levels of sRANKL and CTX (Additional file 1: Supplementary Figures 4a-c) as well as reduced TRAP-positive MNCs and mRNA levels of
Rank, Nfatc1 and Ctsk (Additional file 1: Supplementary Figures 5a–c). Moreover, to investigate whether RNAs within SHED-EVs play a role in their therapeutic effect, we systemically infused OVX mice with SHED-EVs pretreated with or without RNase for 30 min at 37 °C and examined the osteoporotic phenotype after four weeks. RNase-pretreated SHED-EVs showed no significant recovery of the osteoporotic phenotype, differentiation, or function of osteoclast-lineage cells (Figure 3, Additional file 1: Supplementary Figures 4, 5). These findings indicate that RNAs, specifically miRNAs, in SHED-EVs contribute to bone loss rescue in OVX mice.

Systemic administration of SHED-EVs rescue impaired BMMSC function via telomerase activity in recipient OVX mice

We next examined the effect of SHED-EV infusion on impaired BMMSCs in recipient OVX mice and found that recipient BMMSCs recovered Tert mRNA levels and telomerase activity, as determined via RT-qPCR and RQ-TRAP, respectively (Figures 4a, 4b). Furthermore, infusion of SHED-EVs improved CFU-F formation, cell proliferation, as well as CD146 and CD73 expression in recipient BMMSCs, as detected by the CFU-F, BrdU-labeling, and FCM assays, respectively (Figures 4c-4e). Infusion of SHED-EVs also markedly rescued impaired osteogenesis as indicated by the increase in mineralized nodule formation and mRNA levels of Runx2, Bglap, and Sema3a by Alizarin Red staining and RT-qPCR under osteogenic conditions (Figures 4f-4i). However, RNase-treated SHED-EVs did not rescue colony formation, cell proliferation, telomerase activity, osteogenesis, or Sema3a expression in recipient BMMSCs (Figure 4).

SHED-EVs ameliorate the properties of hBMMSCs

To determine the direct effects of SHED-EVs on BMMSCs in mediating cell-cell communication, we incubated hBMMSCs with or without SHED-EVs. Fluorescence microscopy and FCM revealed uptake of SHED-EVs into hBMMSCs (Figures 5a, 5b). SHED-EVs significantly increased the mRNA levels of TERT and telomerase activity when compared to that in MOCK-hBMMSCs, by RT-qPCR and RQ-TRAP, respectively (Figures 5c, 5d). FCM and BrdU results showed that SHED-EVs upregulated the expression of CD146 and cell proliferation, respectively (Figures 5e, 5f). SHED-EVs also enhanced the in vitro osteogenic functions of hBMMSCs, as indicated by increased mineralized nodule formation...
and mRNA levels of *RUNX2*, *BGLAP*, and *SEMA3A* as determined via Alizarin Red staining and RT-qPCR under osteogenic conditions (Figures 5g–5j).

Lastly, to examine whether RNAs from SHED-EVs regulate SHED-EV-mediated enhancement of hBMMSC properties, we assayed hBMMSCs incubated with RNase-pretreated SHED-EVs and found that RNase-pretreated SHED-EVs attenuated the SHED-EV-mediated rescue of *TERT* mRNA levels, telomerase activity, CD146 expression, and cell proliferation in hBMMSCs (Figures 6a–6d). RNase-pretreated SHED-EVs also attenuated SHED-EV-mediated rescue of mineralized nodule formation, upregulation of *RUNX2*, *BGLAP*, and *SEMA3A* mRNA levels, and enhanced *de novo* bone formation in hBMMSCs (Figures 6e–6h). Finally, SHED-EV-pretreated hBMMSCs exhibited a significant increase in bone formation when implanted into immunocompromised mice subcutaneously using HA/TCP as a carrier (Figures 6i, 6j). RNase-pretreated SHED-EVs also attenuated enhanced *de novo* bone formation in hBMMSCs (Figure 6i, 6j).

**Discussion**

Recent studies have shown that the major therapeutic benefit of MSC transplantation for osteoporosis in mice employs multiple mechanisms using trophic factors secreted from donor MSCs that are not imparted by directly replacing bone-forming cells differentiated from donor MSCs in situ [15, 34]. Cytokine/chemokine secretion and cell-cell interactions have been proposed as important in SHED-based therapy in OVX and MRL/lpr mouse models for primary and secondary osteoporosis [12, 15]. However, it is unclear whether SHED-EV-trophic factors participate in SHED-based therapy for osteoporosis. In this study, we have demonstrated that the systemic infusion of SHED-EV improves bone loss in the OVX mouse model for postmenopausal osteoporosis. SHED-EVs also rescued the impaired function of recipient BMMSCs by regulating Tert-associated telomerase activity. Thus, SHED-EVs appear to play a crucial role in the biological crosstalk between donor SHED and recipient BMMSCs to achieve therapeutic effects in postmenopausal osteoporosis.

Epigenetic modifications in bone cells affect the development and therapeutic sensitivity of osteoporosis [35]. An MSC transplantation study showed that MSC-derived exosomes transfer FAS to BMMSCs of recipient FAS-mutated MRL/lpr mice to modify the DNA methylation status of Notch,
resulting in the rescue of impaired recipient BMMSC function [15]. In this study, SHED-EVs effectively recovered the mRNA levels of Tert in the recipient BMMSCs in OVX mice, resulting in improved BMMSC function by regulating telomerase activity and imparting a therapeutic advantage. TERT mRNA levels are controlled by epigenetic modifications, including histone acetylation and methylation, in its promoter region under physiological and pathological conditions [36]. Previous studies have shown that estrogen deficiency increases DNA methylation and downregulates telomerase activity in the bone and peripheral blood cells of postmenopausal women and ovariectomized animals [37–39]. Our data suggest that SHED-EVs may epigenetically modify the OVX-damaged TERT promoter region in BMMSCs in recipient OVX mice. However, we were unable to examine the epigenetic status of the Tert promoter region in the recipient OVX-BMMSCs. Additional experiments are needed to comprehensively examine whether ovariectomy triggers the epigenetic modification of the TERT promoter region and if this is rescued by trophic factor(s) transferred from SHED-EVs into BMMSCs of recipient OVX mice.

Multiple miRNAs regulate the osteogenic differentiation of BMMSCs [40]. For example, microRNA-218-5p targets COL1A1 to promote osteogenic differentiation of OVX-BMMSCs in vitro [41]; while microRNA-145 suppresses the osteogenic differentiation of human BMMSCs [42]. In this study, RNase-pretreated SHED-EVs attenuated the SHED-EV-mediated rescue of Tert-associated telomerase activity in recipient BMMSCs. Therefore, we speculated that the RNA, most likely miRNA, within SHED-EVs affect TERT-stimulated telomerase activity to increase cell proliferation and osteogenic function of murine and human BMMSCs. However, the precise miRNA(s) responsible for modifying TERT gene expression in SHED-EVs remain to be identified. Since miRNAs are evolutionally conserved [43, 44], murine and human models may share miRNA(s) capable of stimulating TERT gene expression in BMMSCs and SHED-EVs.

MSC-secreted exosomes exert therapeutic effects on cell-cell communications in multiple ways [34, 45]. In this study, we identified that SHED-EV-infusion rescued Sema3a mRNA levels in BMMSCs of recipient OVX mice. SEMA3A is an osteoprotective factor produced by osteoblasts [46] and inhibits osteoclast differentiation, while promoting osteoblastic bone formation, leading to the recovery of
bone volume in OVX mice. Further, overexpression of Sema3A mRNA promotes cell proliferation and osteogenic differentiation of BMMSCs [47]. Therefore, our findings propose that SHED-EV-mediated epigenetic modification in recipient OVX mice BMMSCs employs a multi-step approach for therapeutic efficacy; in the first step, SHED-EV-triggered telomerase activity rescues cell proliferation and osteogenic function of recipient BMMSCs. In the second step, SHED-EV-mediated telomerase activity induces secretion of SEMA3A from recipient BMMSCs into the bone microenvironment. This secondary factor then induces bone regeneration by inhibiting osteoclast-mediated bone resorption and enhancement of osteoblast-mediated bone formation in the bone microenvironment of OVX mice.

Recent SHED transplant studies have shown that trophic factor secretion and cell-cell interactions are responsible for immune therapy against osteoporosis [12, 15]. Here we focused on impaired recipient BMMSCs as a therapeutic target and showed that systemic SHED-EV-infusion rescued impaired recipient BMMSCs by regulating TERT-mediated telomerase activity upon transferring RNAs via SHED-EVs. Since SHED-EVs contain a variety of small RNAs, including miRNAs, that are important in systemic cell-cell communication, suggesting that multiple RNAs contribute to the therapeutic potential for osteoporosis in the recipient. However, we cannot exclude the off-target effects exerted on other types of cells in the bone, bone marrow, as well as other tissues and organs. Hence, additional experiments are required to examine whether SHED-EV infusion induces off-target effects in other cells in OVX mice.

Conclusions

Taken together, this study demonstrates that systemic SHED-EV infusion achieved therapeutic efficacy in postmenopausal osteoporosis by targeting recipient BMMSCs and epigenetically rescuing telomerase activity by trophic factor(s), specifically miRNA, within the EVs, resulting in bone regeneration. We have revealed the important interaction between telomerase activity and recipient BMMSCs via SHED-released EVs. Our findings expand the current understanding on the mechanism of SHED-based therapy via SHED-secreted EVs and provide new insights into EV-mediated cell-cell communication in SHED-based therapy. Additional investigation is necessary to determine the effector miRNA(s) in SHED-EVs and their epigenetic modification required to rejuvenate impaired
recipient BMMSCs by controlling telomerase activity.

Supplementary Information

Additional file 1: Supplementary Methods. Supplementary Table 1. Specific antibodies for flow cytometry and western blotting. Supplementary Table 2. TaqMan probes used for the mouse genes. Supplementary Table 3. TaqMan probes used for the human genes. Supplementary Figure 1. Systemic SHED transplantation improved bone loss in ovariectomized mice (OVX mice).

Supplementary Figure 2. Systemic SHED transplantation reduced the enhanced osteoclast activity in OVX mice. Supplementary Figure 3. Systemic SHED transplantation suppressed in vitro osteoclast differentiation of OVX mouse-derived bone marrow cells (BMCs). Supplementary Figure 4. Systemic SHED-EVs administration reduced the enhanced osteoclast activity in OVX mice.

Supplementary Figure 5. Systemic SHED-EVs administration suppressed in vitro osteoclast differentiation of OVX mouse-derived bone marrow cells (BMCs).

Abbreviations

Bglap: Bone gamma carboxyglutamic acid protein; BMD: Bone mineral density; BMMSCs: Bone marrow mesenchymal stem cells; BrdU: Bromodeoxyuridine; BV/TV: Trabecular bone volume versus total volume; CFSE: Carboxyfluorescein diacetate succinimidyl ester; CFU-F: Colony forming units-fibroblast; CM: Conditioned medium; Ctsk: Cathepsin K; CTX: C-terminal telopeptides of type I collagen; ELISA: Enzyme-linked immunosorbent assay; EVs: Extracellular vesicles; FCM: Flow cytometry; HA/TCP: Hydroxyapatite tricalcium phosphate; hBMMSCs: Human bone marrow mesenchymal stem cells; L3: Third lumbar vertebrae; mBMMSCs: Mouse bone marrow mesenchymal stem cells; micro-CT: Micro-computed tomography; miRNAs: MicroRNAs; MNCs: Multinuclear cells; MSCs: Mesenchymal stem cells; Nfatc1: Nuclear factor of activated T-cell; OVX-BMMSCs: BMMSCs derived from OVX mice; OVX: Ovariectomy-induced; PBS: Phosphate-buffered saline; Rank: Receptor activator for nuclear factor κB; Rnase: Ribonuclease A; RQ-TRAP: Quantitative telomerase repeated amplification protocol; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; Runx2: Runt-related transcription factor 2; Sema3a: Semaphorin-3a; SHED: Stem cells from human exfoliated deciduous teeth; SHED-BMMSCs: SHED transplanted mice derived bone marrow mesenchymal stem...
cells; SHED-EVs: SHED-releasing extracellular vesicles; sRANKL: Soluble receptor activator for nuclear factor κB ligand; Tb.N: Trabecular numbers; Tb.Th: Trabecular thickness; TERT: Telomerase reverse transcriptase; TRAP: Tartrate-resistant acid phosphatase

Declarations

Ethics approval and consent to participate

Human deciduous teeth were collected from discarded clinical samples from healthy pediatric donors (5–7 years) in the Department of Pediatric Dentistry, Kyushu University Hospital. Human sample handling was approved by Kyushu University Institutional Review Board for Human Genome/Gene Research (Protocol Number: 393-01). We obtained written informed consent from the parents of each patient on behalf of the child donors. All experimental procedures in this study were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no competing financial interests.

Funding

This work was supported by the Grants-in-Aid for Scientific Research (B) (JSPS KAKENHI Grant Number 25293405 to T.Y.) and Early-Career Scientists (JSPS KAKENHI Grant Number JP 19K18945 to S.S.) of Japan Society for Promotion of Science.

Author’s Contributions

Contribution: S.S., collection and assembly of data, data analysis, and interpretation, and critical revision of manuscript; S.M., collection and assembly of data, data analysis, and interpretation; K.N., H.K., N.U., Y.K., H.Y., T.K., data analysis and interpretation; T.Y., collection and assembly of data, data analysis and interpretation, conception and design, data analysis and interpretation, manuscript
writing, and critical revision of manuscript.

**Acknowledgments**

We would like to thank Editage (www.editage.com) for English language editing.

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Figures
Systemic transplantation of stem cells from human exfoliated deciduous teeth (SHED) rescued the properties and functions of mouse bone marrow mesenchymal stem cells (mBMMSCs) in recipient ovariectomized (OVX) mice. OVX mice were intravenously
administered SHED two days post-surgery and harvested four weeks after transplantation. (a) The graph shows the number of adherent colonies formed by the colony-forming unit fibroblasts (CFU-F) assay. (b) Proportion of bromodeoxyuridine-positive (BrdU+) cells in mBMMSCs. (c) Proportion of cells positive for each cell surface marker in mBMMSCs as determined by flow cytometric (FCM) analyses. (d–g) Representative images of mineralized nodules were detected by Alizarin Red staining four weeks after inducing osteogenesis (d).

Alizarin Red-positive (Alizarin Red+) areas (e). Ratio of the expression of osteogenic markers including Runt-related transcription factor 2 (Runx2) and bone gamma carboxyglutamic acid protein (Bglap) (f) and osteoprotective marker Semaphorin-3a (Sema3a) (g) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) one week after osteogenesis induction. (h) Telomerase activity in mBMMSCs as represented by the threshold cycle values (Ct) obtained by the real-time quantitative telomerase repeated amplification protocol (RQ-TRAP). 293T, HEK 293T cells; 293T H.l., heat-inactivated HEK 293T cells. (i) The expression of Tert in mBMMSCs. (j) Representative fluorescent micrographs of the bone marrow of OVX mice seven days after SHED infusion as seen by the cell tracking assay using carboxyfluorescein diacetate succinimidyl ester or phosphate-buffered saline (PBS; MOCK) pretreated SHED. a–h: Sham, sham-operated group; OVX, PBS-infused OVX group; SHED, SHED-transplanted OVX group. a–c, e–i: n = 7 for all groups. *P < 0.05, **P < 0.01, ***P < 0.005, ns: no significance. Graph bars represent mean ± standard error of mean (SEM). d, j: Bars = 10 mm (d), 100 µm (j). f, g, i: The data are shown as the ratio of the samples to the mBMMSCs derived from sham-operated mouse (Sham = 1).
Characterization of SHED-derived extracellular vesicles (SHED-EVs). SHED-EVs were isolated from SHED conditioned medium harvested from three-day cultures. (a) Representative histogram of the particle size of SHED-EVs by nanotacking particle analysis. (b) Representative histograms for the FCM-based analysis of the expression of CD9, CD63, CD81 and CD90 in SHED-EVs. PE, R-phycoerythrin. Areas filed with red, target antibody-stained histograms; solid lines, isotype-matched control-stained histograms. (c) Representative western blotting images for the expression of CD63, CD81, and calnexin (CANX) in SHED and SHED-EVs (d). Representative histograms of the small RNA and micro
RNA (miRNA) content within SHED-EVs. (e, f) SHED-EVs were treated with RNase A (5 U/mL; RNase) or PBS (MOCK) for 30 min. Representative histograms of the small RNA and miRNA content in SHED-EVs (e) and particle size of SHED-EVs (f).
Systemic SHED-EV infusion improved bone loss and enhanced osteoclast activity in OVX mice. OVX mice were intravenously administered SHED-EVs (100 µg/mouse) pretreated with or without RNase for 30 min two days post-surgery and harvested four weeks after infusion. (a–c) Representative trabecular bone structure of the third lumber vertebra (L3) as seen by the micro-computed tomography (micro-CT) assay (a). The graphs show BMD (b) and BV/TV, Tb.N, and Tb.Th (c) of the trabecular bone in L3. a–c: Sham, sham-operated group; OVX, PBS-infused OVX group; EV, MOCK-treated SHED-EV-infused OVX group; RNase-EV, RNase-retreated SHED-EV-infused OVX group. a: Bars = 1 mm. b, c: n = 7 for all groups. *P < 0.05, **P < 0.01, ***P < 0.005, ns: no significance. Graph bars represent mean ± standard deviation (SD).
Systemic SHED-EV infusion rescued the properties and functions of mBMMSCs in recipient OVX mice. (a) Telomerase activity in mBMMSCs represented by their Ct values using the RQ-TRAP assay. 293T, HEK 293T cells; 293T H.I., heat-inactivated HEK 293T cells. (b) Number of adherent colonies as detected by the CFU-F assay. (c) Proportion of BrdU+ cells. (d) Proportion of cells positive for each surface marker in mBMMSCs as determined by FCM analysis. (e–h) Representative images of mineralized nodules after Alizarin Red staining four weeks after osteogenesis (e). The Alizarin Red+ cells. Bars = 10 mm (d) (f). The ratio of the expression of Runx2 and Bglap (g) and Sema3a (h) by RT-qPCR one week after the induction
of osteogenesis. The results have been expressed as a ratio to the expression in the control BMMSCs derived from sham-operated mouse (Sham = 1). a–i: Sham, sham-operated group; OVX, PBS-infused OVX group; EV, MOCK-treated SHED-EV-infused OVX group; RNase-EV, RNase-retreated SHED-EV-infused OVX group. a–e, g–i: n = 7 for all groups. *P < 0.05, **P < 0.01, ***P < 0.005, ns: no significance. Graph bars represent mean ± SEM.

Figure 5

SHED-EVs enhanced Tert-associated telomerase activity and osteogenic functions of human
BMMSCs (hBMMSCs). hBMMSCs were incubated with SHED-EVs (EV; 20 µg) or PBS (MOCK) for 3 days. (a, b) hBMMSCs were incubated with SHED-EVs loaded with SHED-EVs labeled with CFSE and PBS for 3 days. Representative fluorescent images of hBMMSCs are shown. Nuclei were stained with 4′,6-diamidino-2-phenylindole (a). Representative histograms of hBMMSCs loaded with CFSE-EV and MOCK-EV after FCM analysis. Numbers indicate the means of the positive cells (b). (c) Expression of human TERT in hBMMSCs by RT-qPCR. (d) Telomerase activity in hBMMSCs as detected by RQ-TRAP. 293T, HEK 293T cells; 293T H.I., heat-inactivated HEK 293T cells. (e) Expression of CD146 in hBMMSCs by FCM. (f) Proportion of BrdU+ cells in hBMMSCs. (g-j) Representative images of mineralized nodules after Alizarin Red staining four weeks after osteogenesis (g). Percentage of the Alizarin Red+ area in the dish area (h). Ratio of the expression of RUNX2 and BGLAP (i) and SEMA3A (j) by RT-qPCR one week after the induction of osteogenesis. a, b: CFSE-EV, CFSE-pretreated SHED-EV-loaded group; MOCK-EV, PBS-pretreated SHED-EV-loaded group. c-j: MOCK, MOCK-pretreated group; EV, hSHED-EV-treated group. k–T: MOCK, MOCK-treated group; MOCK-EV, MOCK-pretreated hSHED-EV-treated group; RNase-EV, RNase-pretreated hSHED-EV-treated group. a, g: Bars = 10 mm. b, c-f, h–j: n = 7 for all groups. *P < 0.05, **P < 0.01, ***P < 0.005. ns: no significance. Graph bars represent mean ± SEM (b, c-f, h–j) and mean ± SD (l). c, i, j: The results are shown as a ratio to the expression in MOCK-treated group (MOCK = 1).
RNAs from hSHED-EVs regulated hSHED-EV-enhanced TRET-associated hBMMSC properties and functions. (a–h) hBMMSCs were incubated with or without RNase-pretreated and MOCK-pretreated SHED-EVs (RNase-EV and MOCK-EV, respectively). Expression of TERT in
hBMMSCs (a). Telomerase activity in hBMMSCs assayed by RQ-TRAP. H.I., heat-inactivated group (b). Expression of CD146 in hBMMSCs after FCM analysis (c). Percentages of BrdU+ cells in mBMMSCs (d). SHED-EVs were incubated osteogenic conditions. Representative images of mineralized nodules after Alizarin Red staining four weeks after osteogenesis (e). Percentage of Alizarin Red+ area in dish area (f). Ratio of the expression of RUNX2 and BGLAP (g) and SEMA3A (h) as detected by RT-qPCR one week after the induction of osteogenesis. (i, j) hBMMSCs (4 × 10⁶) were introduced with hydroxyapatite tricalcium phosphate (HA/TCP) into immunocompromised mice. Representative histological images of de novo bone tissue (B) in the implants are shown after hematoxylin and eosin staining (i). Area of de novo bone in the implants (j). a–j: MOCK, MOCK-treated group; MOCK-EV, MOCK-pretreated hSHED-EV-treated group; RNase-EV, RNase-pretreated hSHED-EV-treated group. e, i: Bars = 10 mm (e), 10 µm (i). a–d, f, g, h, j: n = 7 for all groups. *P < 0.05, **P < 0.01, ***P < 0.005. ns: no significance. Graph bars represent mean ± SEM (a–d, f, g) and mean ± SD (j). a, g, h: The results are shown as a ratio to the expression in MOCK-pretreated hSHED-EV-treated group (MOCK-EV = 1, respectively).

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