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Mesenchymal stromal cell exosomes enhance dental pulp cell functions and promote pulp-dentin regeneration

Jiajun Shi[a], Kristeen Ye Wen Teo[a], Shipin Zhang[a], Ruenn Chai Lai[b], Vinicius Rosa[a,c], Huei Jinn Tong[a], Mandeep S. Duggal[d], Sai Kiang Lim[b], Wei Seong Toh[a,e,f,g,h,*]

[a] Faculty of Dentistry, National University of Singapore, Singapore
[b] Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore
[c] Oral Care Health Innovations and Designs Singapore, National University of Singapore, Singapore
[d] College of Dental Medicine, QU Health, Qatar University, Qatar
[e] Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[f] Department of Biomedical Engineering, Faculty of Engineering, National University of Singapore, Singapore
[g] Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore
[h] Integrative Sciences and Engineering Program, NUS Graduate School, National University of Singapore, Singapore

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ABSTRACT

Mesenchymal stromal/stem cell (MSC) therapies are currently being explored for dental pulp regeneration. As the therapeutic effects of MSCs in tissue repair are mediated mainly through the release of extracellular vesicles (EVs) including exosomes, we investigated here the cellular processes and molecular mechanisms modulated by MSC exosomes in dental pulp regeneration. Using dental pulp cell (DPC) cultures, we demonstrated that MSC exosomes could increase DPC migration, proliferation, and odontogenic differentiation. The enhancement of these cellular processes was mediated through exosomal CD73-mediated adenosine receptor activation of AKT and ERK signaling. Consistent with these observations, MSC exosomes increased the expression of dentin matrix proteins and promoted the formation of dentin-like tissue and bridge-like structures in a rat pulp defect model. These effects were comparable to that of mineral trioxide aggregate (MTA) treatment. MSC exosomes also yielded recellularized pulp-dentin tissues in the root canal of endodontically-treated human premolars, following subcutaneous implantation in the mouse dorsum. Together, our findings suggest that MSC exosomes could exert a multi-faceted effect on DPC functions including migration, proliferation and odontogenic differentiation to promote dental pulp regeneration. This study provides the basis for development of MSC exosomes as a cell-free MSC therapeutic alternative for pulp-dentin regeneration.

1. Introduction

Dental pulp is a vascularized dental tissue embedded in mineralized structures, including dentin, enamel, and cementum, and maintains the homeostasis and viability of the tooth [1,2]. Dental caries or trauma may affect the pulp and lead to irreversible pulpitis and pulpal non-vitality. Currently, the standard-of-care involves pulp extirpation, instrumentation, and disinfection of the root canal system, followed by root canal obturation. However, this procedure devitalizes the tooth and increases its susceptibility to post-operative fracture and re-infection due to coronal leakage or microleakage [3,4]. Furthermore, this results in premature arrest of root development in immature teeth with open apices [5].

Dental pulp tissue engineering employing the use of stem cells, biomaterials, and growth factors has emerged as a promising strategy in regenerative endodontics to replace the damaged tissues and to restore functions of previously necrotic and infected root canals [6]. Of note, there is a strong interest in pulp-dentin regeneration via delivery of mesenchymal stromal/stem cells (MSCs) such as dental pulp stem cells (DPSCs) [7,8]. However, cell-based MSC therapies are facing hurdles in clinical implementation due to high costs and operational challenges in maintaining the optimal viability and potency of cells needed for transplantation [9].

It is now widely accepted that MSCs mediate tissue repair mainly through paracrine secretion [10]. Among the broad spectrum of trophic factors secreted by MSCs, extracellular vesicles (EVs), particularly small EVs of size 50 – 200 nm that include exosomes have been reported to be therapeutically efficacious against many diverse diseases [11–17]. Several EV types have been described to date, and they include exosomes,

* Corresponding author at: Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, 1E Kent Ridge Road, NUHS Tower Block, Level 11, Singapore.
E-mail address: tohws@nus.edu.sg (W.S. Toh).

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microvesicles, microparticles, exosome-like particles and apoptotic bodies [18]. Due to the significant size overlap and the lack of specific markers, current EV preparations are heterogenous. We have previously reported that our MSC-EV preparations contain at least three distinct EV populations, including exosomes derived from the endosomes [19]. Therefore, we use the term "MSC exosomes" for our MSC-EV preparations.

MSC exosomes have demonstrated therapeutic efficacy for dental pulp regeneration in many animal studies [20–25]. Of note, exosomes derived from dental tissues such as the dental pulp and exfoliated deciduous teeth have been shown to promote pulp regeneration by enhancing cellular processes such as angiogenesis and odontogenesis [23–25]. For instance, exosomes derived from dental pulp tissue were found to promote dental pulp-like tissue regeneration by enhancing migration, proliferation and differentiation of stem cells from the apical dental papilla (SCAPs) [24]. Other studies have also reported enhanced therapeutic effects of exosomes from mineralizing or LPS-preconditioned DPSCs in dental pulp regeneration [20–22]. However, most of these studies have been performed using human tooth fragments subcutaneously implanted to the back of the mice [20,23-25] that may not fully resemble the dental pulp microenvironment. The cellular processes and mechanisms mediated by MSC-EVs in pulp regeneration also remain to be fully elucidated.

In this study, we aimed to investigate the potential of MSC exosomes for pulp-dentin regeneration, and to further decipher the effects of MSC exosomes on key DPC functions, including DPC migration, proliferation and odontogenic differentiation, and the underlying mechanisms, relevant to pulp-dentin regeneration.

2. Methods

2.1. Preparation and characterization of MSC exosomes

MSC exosomes were prepared from an immortalized E1-MYC 16.3 human embryonic stem cell (hESC)-derived MSC line [26]. Briefly, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermofisher Scientific). For exosome preparation, the conditioned medium (CM) was prepared by growing 80% confluent cells in a chemically defined culture medium composed of DMEM (Thermofisher Scientific) supplemented with 1% non-essential amino acids, 1% glutamine, 1% insulin-transferrin-selenium-X, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 5 ng/ml fibroblast growth factor (FGF)-2 (Thermofisher Scientific) and 5 ng/ml platelet-derived growth factor (PDGF)-AB (Cytolab Ltd, Rehovot, Israel) for 3 days [27]. The CM was size-fractionated by tangential flow filtration and concentrated 50 × using a membrane with a molecular weight cut-off of 100 kDa (Sartorius, Göttingen, Germany) [11]. The exosome preparation was assayed for protein concentration using Coomassie Plus (Bradford) protein assay (Thermofisher Scientific), particle size distribution and concentration by ZetaView (Particle Metrix GmbH, Meerbusch, Germany) and CD73/ecto-5′-nucleotidase (NT5E) activity using a PiColorLock™ Gold Phosphate Detection System (Innova Biosciences, Cambridge, UK) in accordance with the MISEV2018 guidelines [28] and more specifically with the identity and potency metrics proposed for MSC-sEV preparations [29,30]. This exosome preparation protocol has been used for preparation of over 100 batches of exosomes with a high batch reproducibility in their protein and particle concentrations, modal diameter and CD73/NT5E activity. In addition to these tests, many of the batches were routinely analysed and confirmed to carry exosome-associated markers including CD81, ALIX and TSG101 [11,14]. For this study, the 98th batch of MSC exosome preparation (Batch AC98) was used. AC98 has a protein concentration of 1.726 mg/ml, particle concentration of 1.45×10^{11} particles/ml, particles of a modal size of 122.7 nm (Fig. S1) and CD73/NT5E activity of 20.67 ± 1.61 mU/μg protein. MSC exosomes were 0.22-μm filtered and stored in -20°C freezer until use.

2.2. Isolation and culture of rat dental pulp cells

Dental pulp cells (DPCs) were isolated from the extracted incisors of 8-week-old female Sprague-Dawley rats following an established protocol with modification [31]. Briefly, the gingival soft tissues were excised and incisors were then extracted using forceps. The incisors were washed with phosphate-buffered saline (PBS) supplemented with 2% penicillin/streptomycin (PS; Thermofisher Scientific). After removing the soft tissues from the root surface, the incisors were split open and the pulp tissues were carefully extracted. These pulp tissues were cut into small pieces and digested in 2% dispase II and 0.2% collagenase I (Worthington, Lakewood, NJ, USA) at 37°C for 1 h. The cell suspension was passed through a 40-μm cell strainer (Corning®, Corning, NY, USA) to disperse into single cells before seeding at a density of 2×10^4 cells/cm^2. The cells were grown in complete culture medium composed of DMEM-F12 supplemented with 10% FBS and 1% PS with medium change every alternate day. Upon confluence, DPCs were dissociated with TrypLe™ (Thermofisher Scientific) and sub-cultured to passage (P) 3 for subsequent experiments.

2.3. Cell viability and proliferation

Rat DPCs were seeded at 5000 cells/well in 96-well plates and treated with 1, 5 and 10 μg/ml of exosomes or vehicle (PBS). At 4, 24 and 48 h, cell viability and DNA content were measured. Cell viability was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit (Promega, Madison, WI, USA). 20 μl of MTS reagent was added to each well and incubated at 37°C for 2 h. Absorbance readings were then taken at 490 nm and 650 nm (reference) using a microplate reader (Infinite® 200 PRO, Tecan™, Männedorf, Switzerland). Total DNA content that is reflective of cell number was measured. Briefly, cells in each well were lysed by 50 μl CelLytic M cell lysis buffer (Sigma, Saint Louis, MO, USA) and the DNA concentration was measured using the Quant-iT™ Picogreen® dsDNA assay kit (Thermofisher Scientific) following the manufacturer's instruction. The fluorescence readings were taken at excitation 480 nm and emission 520 nm using the microplate reader (Infinite® 200 PRO).

2.4. Apoptosis assay

Rat DPCs were seeded at a density of 80,000 cells/well in 6-well plates and treated with either 10 μg/ml of exosomes or PBS. After 48 h, cell apoptosis was assessed using the Dead Cell Apoptosis kit (Thermofisher Scientific) with Annexin V and propidium iodide (PI), following the manufacturer's protocol. Briefly, cells were suspended in 100 μl binding buffer containing 5 μl of Alexa Fluor™ 488 Annexin V and 1 μl of 100 μg/ml PI, followed by incubation at ambient temperature for 15 min. 400 μl binding buffer was then added to each sample, and the stained cells were analysed using the Fortessa LSR flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v10.8.1 (Tree Star Inc).

2.5. Cell migration

The migration of DPCs in response to exosome treatment was assessed using a transwell system [32]. Briefly, 5×10^4 cells in 300 μl low serum culture medium (DMEM-F12 supplemented with 0.5% FBS and 1% PS) were seeded in the upper chamber, and 1, 5 or 10 μg/ml exosomes or PBS were added to the lower chamber. After 16 h, the upper surface of the filter was swabbed free of cells. Cells on the underside of the filter were fixed in 4% paraformaldehyde (PFA) and stained with haematoxylin and eosin. The cells in five randomly selected fields at 100 × magnification were counted. The percentage of cell migration was calculated by normalizing the number of migrated cells to the ini-

tial number of cells seeded and compared against that of the control set at 100%.

2.6. Odontogenic differentiation

Cells were seeded at 3×10^4 cells/well in 24-well plates in complete culture medium for 24 h before medium change to odontogenic medium (OM) comprising of DMEM-high glucose supplemented with 10% FBS, 1% PS, 50 μg/ml ascorbic acid-2 phosphate, 10 mM β-glycerophosphate, 1 mM sodium pyruvate and 100 nM dexamethasone (Sigma) [33]. Cells were treated with 1, 5 and 10 μg/ml exosomes or PBS for up to 7 days with medium change every alternate day.

2.7. Exosome treatment and inhibitor study

P3 DPCs were seeded at a density of 2×10^4 cells/cm^2 and cultured for 16 h before the medium was changed to low serum culture medium for 24 h, and then treated with 1, 5, 10 μg/ml exosomes or PBS for over 48 h. To investigate the involvement of adenosine receptors and activation of AKT and ERK pathways, cells were pre-treated with 1 mM theophylline (a non-selective inhibitor of adenosine receptors) (Sigma) [34], 1 μM wortmannin (AKT inhibitor) or 10 μM U0126 (ERK inhibitor) (Cell Signaling Technology, Danvers, MA, USA) or equivalent volume of distilled water or dimethyl sulfoxide (DMSO) as vehicle controls for 1 h, before treatment with 10 μg/ml exosomes or PBS for 48 h. In experiment with CD73 inhibitor, N^6-Benzyl-α,β-methyleneadenosine 5′-diphosphate disodium salt (PSB12379; Tocris Biosciences, CO, USA), cells were co-treated with exosomes and 10 nM PSB12379 for 1 h. Following treatment, cells were rinsed with PBS and harvested for analysis. In odontogenic differentiation experiments, cells were treated with 10 μg/ml exosomes or PBS in odontogenic medium with or without the inhibitors. Medium change with fresh exosomes and inhibitors was performed every alternate day till day 7.

2.8. Alizarin red S staining

Alizarin red S (ARS) staining was used to detect calcium deposition. Briefly, cells were fixed in 4% PFA, and then stained with 40 mM ARS solution (Sigma) for 10 min. Images were taken using bright-field microscopy (IX70, Olympus, Tokyo, Japan). For semi-quantitative analysis of calcium deposition, the dye was eluted using 10% acetic acid (Sigma) for 30 min and then transferred to a 96-well plate for absorbance reading at 405 nm using the microplate reader (Infinite® 200 PRO).

2.9. Immunofluorescence staining

Immunofluorescence staining was performed to detect dentin matrix proteins including dentin sialophosphoprotein (DSPP) and dentin matrix protein (DMP)1. Briefly, cells were rinsed with PBS and fixed with 4% PFA before being permeabilized with 0.3% Triton X-100. After blocking for 2 h, DPCs were incubated with mouse anti-DSPP (1:100, Novus Biologicals, CO, USA) or rabbit anti-DMP1 (1:100, Novus Biologicals) at 4°C overnight. Following incubation, cells were rinsed with PBS and incubated with secondary antibodies, Alexa Fluor 594 goat anti-mouse IgG (1:500, Thermofisher Scientific) or Alexa Fluor 488 goat anti-rabbit IgG (1:100, Thermofisher Scientific) for 2 h at ambient temperature. DPCs were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Olympus IX70). Total number of cells by DAPI staining and positively stained cells for DSPP and/or DMP1 in five randomly selected fields at 200 × magnification were counted. The percentage of positively stained cells was calculated by normalizing to the total number of cells.

2.10. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell cultures using PureLink® RNA Mini kit (Thermofisher Scientific) according to manufacturer's instruction. The RNA was reverse transcribed using iScript™ reverse transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was then amplified with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and primers (Table S1) using the CFX Connect™ real-time PCR system (Bio-Rad). The PCR cycling condition comprised an initial denaturation at 95°C for 30 s followed by 40 cycles of amplification consisting of a 15 s denaturation at 95°C and a 30 s extension at 60°C. Relative mRNA expression levels were normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA, and calculated using the comparative ΔCT method [35], and finally expressed as fold changes.

2.11. Western blot hybridization

Western blot hybridization was performed using standard protocols. Briefly, proteins were denatured, separated on 4-12% polyacrylamide gels (Thermofisher Scientific), electro-blotted onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA), probed with primary antibody followed by incubation with horseradish peroxidase (HRP)-coupled secondary antibody against the primary antibody. The primary antibodies included anti-AKT (pan), anti-phospho-AKT (Ser473), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000, Cell Signaling Technology, Beverly, MA, USA), and anti-GADPH (1:10000, Abcam, Cambridge, MA, USA). After incubating with the appropriate HRP-coupled secondary antibodies (GE Healthcare), the protein bands were visualized by incubating with SuperSignal West Pico Chemiluminescent Substrate (Thermofisher Scientific) and documented using the ChemiDoc™ MP System (Bio-Rad).

2.12. Rat dental pulp defect model

Sixteen 8-week-old male Sprague-Dawley rats with a mean weight of 271 ± 27.2 g (range 227 – 305 g) were used in this study. As illustrated in Fig. 4, the surgical procedure was modified from a previously reported protocol [36]. Under general anesthesia, class I defects (1.5 mm diameter × 1.5 mm depth) were surgically created on the occlusal surface of bilateral maxillary first molars using a 1/4 RA carbide bur (Wave Dental, Ningbo, China) and a dental handpiece (NSK, Tokyo, Japan). The procedure was performed under copious PBS irrigation to prevent overheating and to remove tissue debris. Sterile cotton pellets were used to control bleeding and to achieve hemostasis of the pulp. The animals were randomly assigned to four groups (n = 8 defects/group): defects treated with collagen sponge (CS), defects treated with collagen sponge soaked with 2 μl PBS (CS/PBS), defects treated with collagen sponge containing 20 μg exosomes in 2 μl PBS (CS/Exo), and defects treated with collagen sponge coated with mineral trioxide aggregate (CS/MTA). Bovine collagen I/III sponges (HealiAid®, Maxigen Biotech Inc, Taoyuan City, Taiwan) were cut into small pieces (1 mm diameter × 1 mm depth) before the surgery. MTA (Medcem GmbH, Wien, Austria) is commonly used as a pulp-capping material for clinical maintenance of pulp vitality and therefore served as a positive control. All defects were sealed with glass-ionomer cement (GIC; GC Fuji II, Tokyo, Japan). Subcutaneous injections of analgesics (Buprenorphine; 0.2 ml/100 g) were given twice daily for 3 days post-operatively. The animals were housed in pairs and allowed to move without restriction. Standard food and water were provided *ad libitum*. This animal procedure was approved by the Institutional Animal Care and Use Committee (IACUC) at the National University of Singapore under protocol number: R19-0502. At 4 weeks, animals were euthanized and the maxillae harvested for histological analysis.

2.13. Subcutaneously implanted human premolar model

The efficacy of MSC exosomes for pulp-dentin formation was further investigated using a human premolar model [37]. Maxillary or mandibular premolars were collected from 11- to 30-year-old patients undergoing orthodontic treatment with National Healthcare Group Domain Specific Review Board approval (study reference number: 2019/01051). Surrounding soft tissues were removed and premolar roots were cut to ~10 mm in length. Using a barbed broach (000#, Denco, Shenzhen, China), pulp tissues were extirpated, followed by cleaning and shaping of the root canals with hand files (25#, M-Access K-Flies, Dentsply Maillefer, Ballaigues, Switzerland) and irrigation with 1% sodium hypochlorite (Dentalife, Ringwood, Australia). Premolar roots were disinfected with 70% ethanol and were stored at ambient temperature until use. Untreated human premolars served as naive control for the morphology of human dental pulp.

Twelve 7-week-old severe combined immunodeficient (SCID) mice with a mean weight of 21.7 ± 0.4 g (range 20.9 – 22.2 g) were randomly allocated into two groups (n = 6/group): premolar roots treated with collagen gel prepared with PBS (CG/PBS) or collagen gel prepared with exosomes (CG/Exo). Under general anesthesia, mid-longitudinal skin incision of ~12 mm was made on the dorsum of each mouse followed by blunt dissection to form a subcutaneous pocket. 40 μl collagen type I solution (Corning, NY, USA) was mixed with 10 μl of 20 μg exosomes or equivalent volume of PBS containing 0.92 μl of 1 N NaOH (Sigma) to neutralize the acidic collagen solution. The mixture was then injected into the premolar root and gelation was achieved following incubation at 37 °C for 30 min. The premolar construct was then placed inside the subcutaneous pocket and the incision was closed with absorbable sutures (Ethicon, Inc., NJ, USA). After surgery, the animals were allowed to move freely in their cages with access to food and water. The animal procedure was approved by the IACUC at the National University of Singapore under protocol number: R19-1032. At 5 weeks, animals were euthanized and specimens harvested for analysis.

2.14. Histology and immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin (Sigma) overnight and decalcified in 30% buffered formic acid for up to 6 weeks. After decalcification, the samples were dehydrated and embedded in paraffin. Serial sections were cut at 5-μm thickness and stained with haematoxylin and eosin (HE) to examine the general morphology and with Masson's Trichrome (MT) to examine the reparative dentin formation. The stained sections were imaged by an inverted microscope (Olympus IX70). The newly-formed reparative dentin (osteodentin) was demarcated under 100 × magnification and the areas measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentage of reparative dentin tissues in each sample was calculated by normalizing against the total pulp defect area (for rat pulp defect model) or the total root canal area of the premolar (for subcutaneous implanted human premolar model).

Immunohistochemistry was performed using a biotin-streptavidin Lab Vision^{TM} UltraVision^{TM} detection system (Thermofisher Scientific) to detect DSPP and DMP1 [13]. Briefly, the sections were hydrated before subjected to antigen retrieval using sodium citrate buffer (pH 6.0) at 60°C for 1 h. Endogenous peroxidase was quenched using peroxidase block for 15 min, and non-specific protein binding was blocked by incubation with Ultra V blocking solution for 5 min. The mouse anti-DSPP antibody (1:250, Santa Cruz Biotech, Dallas, TX, USA) or rabbit anti-DMP1 antibody (1:500, Biorbyt, Cambridge, UK) was then applied at ambient temperature for 1 h before rinsing with PBS and incubating with biotinylated secondary antibody for 30 min. The antibody-antigen reaction was visualized by applying the 3,3′-diaminobenzidine (DAB) chromogen for 1 min. Nuclear counterstaining was performed using Mayer's haematoxylin (Sigma). The stained sections were imaged using an inverted microscope (Olympus IX70). The percentage of DSPP or DMP1 positively stained area covering the pulp defect region or the root canal space of each tissue sample at 100 × magnification was measured and normalized against the total defect area (for rat pulp defect model) or the total root canal area of the premolar fragment (for subcutaneous implanted human premolar model) using the ImageJ software (National Institutes of Health).

2.15. Statistical analysis

All quantitative data were presented as mean ± standard deviation. Student's t-test or one-way analysis of variance (one-way ANOVA) followed by Scheffe's post-hoc test was performed for normally distributed data, and Mann-Whitney test was performed for non-normal data using SPSS version 25.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of MSC exosomes on DPC functions

We first tested the effects of MSC exosomes on DPC migration, proliferation, and odontogenic differentiation, which are cellular processes relevant to dental pulp regeneration (Fig. 1A). In our cell migration assay, MSC exosomes enhanced DPC migration in a dose-dependent manner (Fig. 1B). At 10 μg/ml exosomes, exosome-treated DPCs exhibited ~1.7-fold higher migration rate than PBS-treated cells ($P < 0.001$, Fig. 1B). As early as 4 h, the viability of DPCs increased markedly, as compared with the PBS control, at 5 and 10 but not 1 μg/ml exosomes ($P < 0.001$, Fig. 1C). The rate of increase at 5 and 10 μg/ml exosomes appeared similar at 4 and 24 h, suggesting that the maximum increase was at 5 μg/ml or less. By 48 h, DPCs treated with 10 μg/ml exosomes demonstrated at least 1.8-fold higher viability than the PBS control ($P < 0.001$, Fig. 1C). Consistent with the increase in cell viability, DNA content which is reflective of the cell number increased with increasing exosome concentrations. At 4 h, MSC exosomes induced significant increase in cell numbers at 1, 5 and 10 μg/ml as compared to the PBS control ($P < 0.001$, Fig. 1D). By 48 h, DPCs treated with 10 μg/ml exosomes proliferated ~1.6-fold faster than those treated with PBS ($P < 0.001$, Fig. 1D). We next examined if increased DPC viability and proliferation with exosome treatment were accompanied by reduced apoptosis. By 48 h, DPCs treated with 10 μg/ml exosomes had higher percentage of live cells ($P < 0.05$) and lower percentage of apoptotic cells ($P < 0.05$) than those treated with PBS (Fig. 1E).

These findings were supported by gene expression analysis (Fig. 1F). Genes associated with DPC survival and proliferation such as *FGF-2, IGF-1* and *Ki67* but not *Survivin* were significantly upregulated at mRNA level within 24 h exposure to exosomes ($P < 0.05$, Fig. 1F). Notably, FGF-2 which regulates DPC migration and proliferation [38] was upregulated as early as 4 h, providing a temporally relevant rationale for our observed exosome enhancements of DPC migration at 16 h and proliferation from 4 h onwards. Another chemotactic factor, SDF-1α was also increased at mRNA level at 4 h, although no changes in *CXCR4* was observed. Consistent with the reduced apoptosis of DPCs observed with exosome treatment (Fig. 1E), there was suppression of *Bax* from 24 h, which was accompanied by upregulation of *Bcl-2* at 48 h with exosome treatment (Fig. 1F).

Next, we examined the effects of MSC exosomes on early DPC odontogenic differentiation. As evidenced by ARS staining and dye elution, 5 and 10 but not 1 μg/ml exosome treatment enhanced odontogenic differentiation and mineralization of DPCs at 7 days, with significantly higher levels of calcium deposition, as compared to PBS treatment ($P < 0.001$, Fig. 2A and C). They also increased the expression of putative odontogenic differentiation markers, DSPP and DMP1, and notably, DSPP and DMP1 double positive DPCs, as compared to cells treated with PBS ($P < 0.01$, Fig. 2B and D). These findings were supported by the significant upregulated expression of genes associated with odontogenic

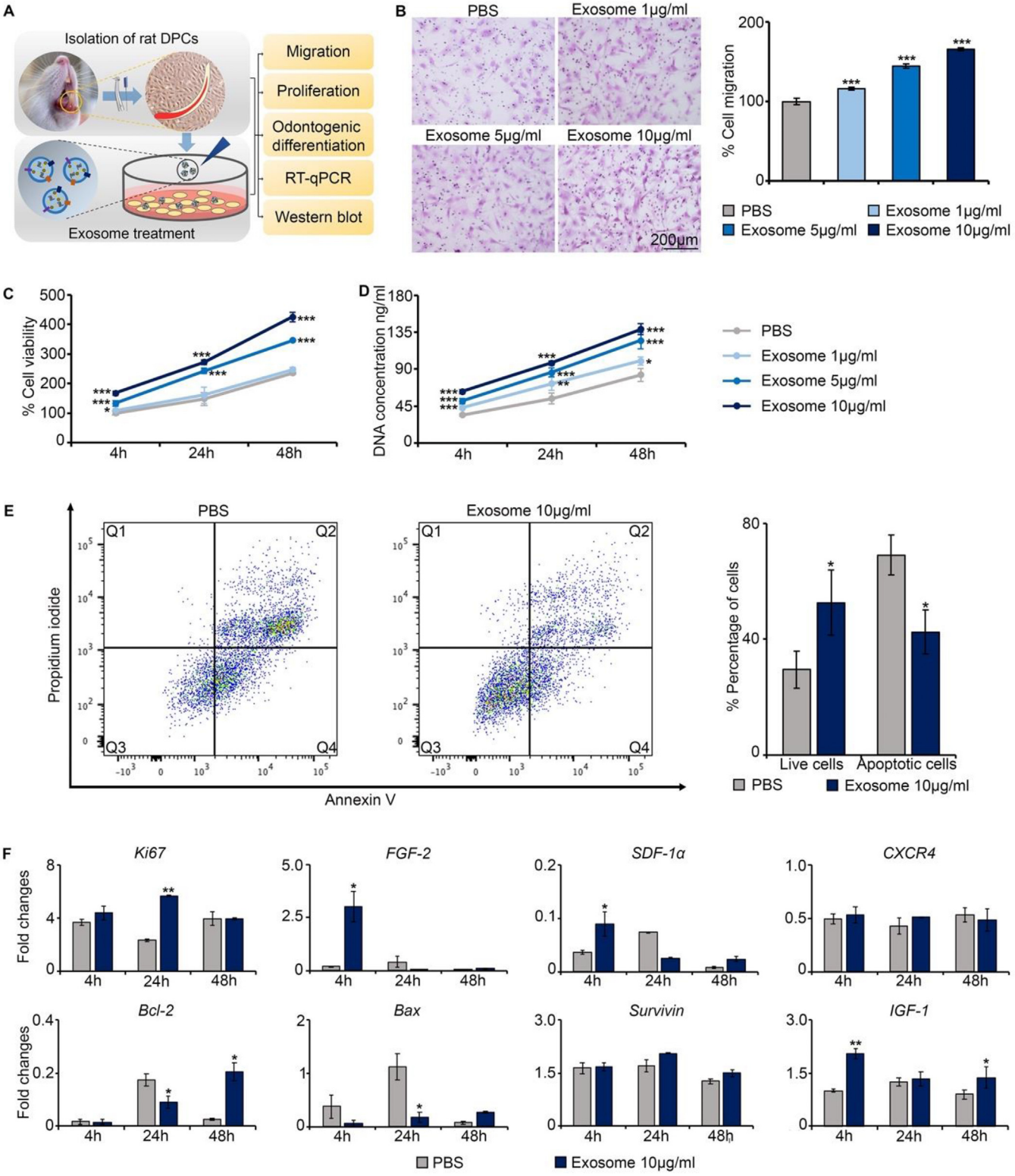

Fig. 1. Effects of MSC exosomes on DPC functions. (A) Experimental design. (B) Transwell migration assay showed that MSC exosomes enhanced DPC migration ($n = 4$). (C) MTS metabolic viability assay and (D) DNA assay showed potent effects of MSC exosomes on DPC viability and proliferation ($n = 5$). (E) Apoptosis assay showed increased percentage of live cells and reduced percentage of apoptotic cells ($n = 4$). (F) RT-qPCR analysis showed regulation of genes associated with DPC survival, proliferation, and migration with 10 μg/ml exosome treatment ($n = 3$). Representative results of 2 independent experiments. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control.

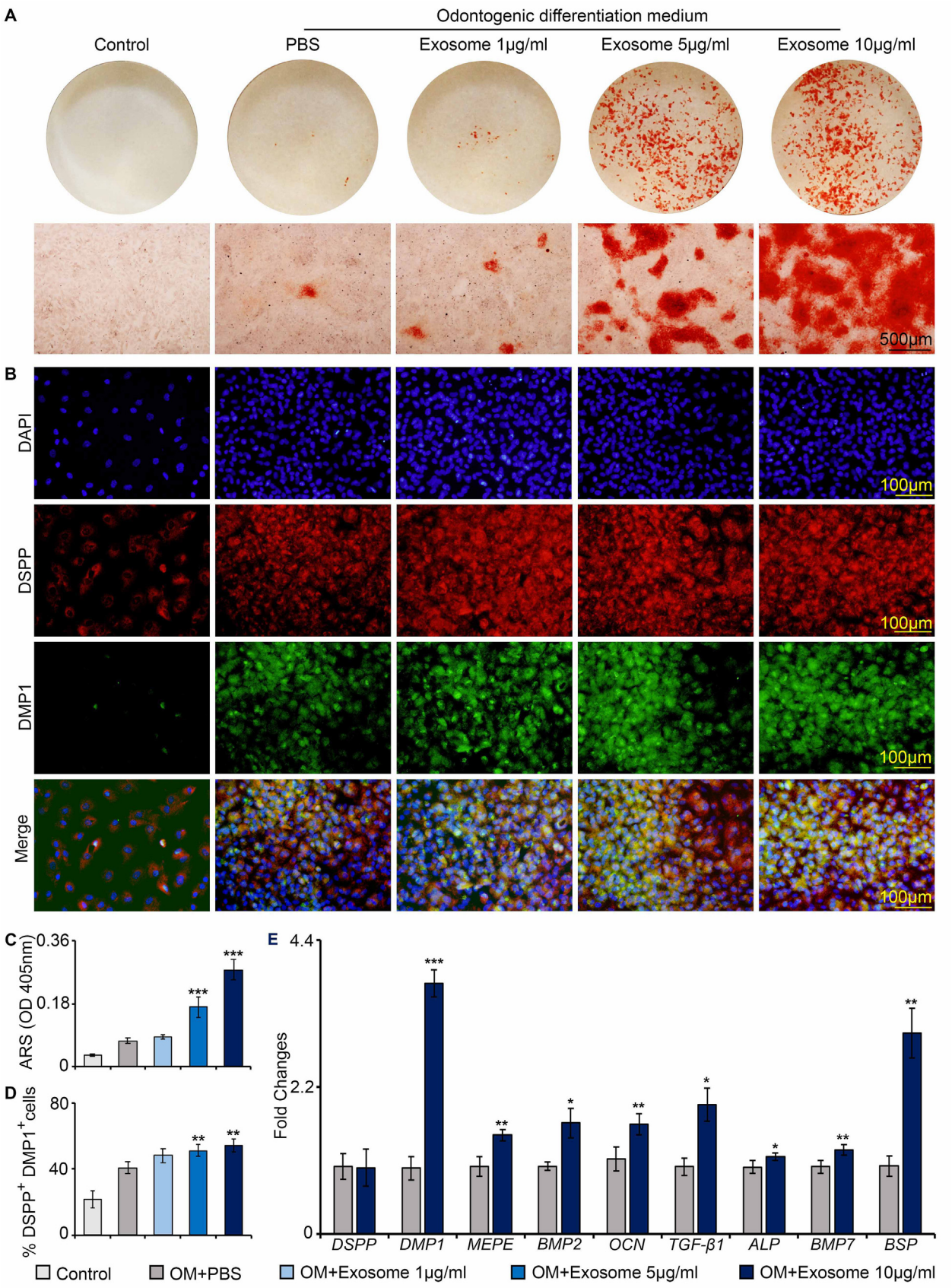

Fig. 2. Effects of MSC exosomes on odontogenic differentiation of DPCs. (A) ARS staining and (C) quantification of eluted dye showed enhanced odontogenic differentiation and mineralization with 5 and 10 μg/ml exosome treatment. (B) Immunofluorescence staining and (D) quantification of DSPP and DMP1 showed significantly higher percentages of DSPP and DMP1 positively stained cells with 5 and 10 μg/ml exosome treatment. (E) RT-qPCR analysis showed regulation of genes associated with odontogenic differentiation with 10 μg/ml exosome treatment. Representative results of 3 independent experiments. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to PBS ($n = 3$).

differentiation, including *DMP1, MEPE, BMP2, OCN, TGF-β1, ALP, BMP7* and *BSP* with exosome treatment as compared with PBS treatment ($P < 0.05$, Fig. 2E).

3.2. MSC exosomes enhance DPC functions through CD73/NT5E-mediated adenosine activation of AKT/ERK signaling pathways

MSC exosomes had been shown to activate AKT and ERK signaling [13,32] partly through exosomal CD73/NT5E that hydrolyzes adenosine monophosphate (AMP) to adenosines which in turn interact with adenosine receptors to elicit AKT and ERK phosphorylation [39]. This adenosine activation of AKT and ERK signaling had promoted cell functions such as migration and proliferation of chondrocytes [32] and periodontal ligament cells [13]. We therefore postulated that CD73/NT5E-mediated adenosine activation of AKT and ERK signaling pathways could similarly be involved in DPC migration, proliferation, and odontogenic differentiation.

We first studied the effect of MSC exosomes on AKT and ERK signaling in DPC culture. Following exposure to 10 μg/ml exosomes, AKT and ERK were rapidly phosphorylated and peaked at 15 min before declining thereafter. Notably, AKT and ERK phosphorylation induced by MSC exosomes was consistently and statistically higher than that of PBS control for up to 60 min ($P < 0.05$, Fig. 3A).

Next, we investigated the involvement of CD73/NT5E in the phosphorylation of AKT and ERK. In the presence of CD73 inhibition by PSB12379, exosome-mediated phosphorylation of AKT ($P < 0.05$) and lesser extent of ERK was attenuated (Fig. 3B). In the presence of theophylline inhibition of adenosine receptors, phosphorylation of AKT and ERK was reduced ($P < 0.05$, Fig. 3C). Exosome-mediated phosphorylation of AKT and ERK was also abrogated by treatment with wortmannin ($P < 0.001$) and U0126 ($P < 0.01$) respectively (Fig. 3E). Functionally, MSC exosomes enhanced DPC migration compared to PBS control ($P < 0.001$, Fig. 3D and F). However, this enhancement was not reversed by adenosine receptor inhibition or ERK inhibition, and only partially attenuated by AKT inhibition ($P < 0.001$, Fig. 3F). Similarly, MSC exosomes enhanced DPC proliferation compared to PBS control ($P < 0.01$, Fig. 3G–H). However, this exosome-mediated DPC proliferation was partially reversed with inhibition of adenosine receptors ($P < 0.01$, Fig. 3G), AKT ($P < 0.05$, Fig. 3H) and ERK signaling ($P < 0.05$, Fig. 3H) within 48 h. Additionally MSC exosomes promoted early odontogenic differentiation and mineralization (Figs. S2A–B and S3A–B) with significantly increased calcium deposition ($P < 0.05$, Figs. S2C and S3C), and DSPP and DMP1 co-expressing cells ($P < 0.01$, Figs. S2D and S3D) compared to PBS control. However, this exosome-mediated odontogenic differentiation with increase in calcium deposition, and DSPP and DMP1 co-expressing cells was partially suppressed with inhibition of adenosine receptors ($P < 0.01$, Fig. S2C–D), AKT ($P < 0.05$, Fig. S3C–D) and ERK signaling ($P < 0.05$, Fig. S3C–D).

3.3. MSC exosomes promote pulp-dentin regeneration in a rat pulp defect model

The *in vitro* findings prompted us to further validate the therapeutic efficacy of MSC exosomes for *in vivo* pulp-dentin regeneration in a rat pulp defect model (Fig. 4). In this model, a 1.5 mm class I defect was created on the occlusal surface of the maxillary first molar, before implanted with a collagen sponge containing exosomes (CS/Exo), PBS (CS/PBS) or MTA (CS/MTA). At 4 weeks post-treatment, 8 of 8 defects in CS/Exo group showed formation of reparative dentin bridges, comparable in extent to that in the CS/MTA group (Fig. 4B–C). In contrast, defects in CS and CS/PBS control groups had mostly collagen remnants with little tissue formation and no apparent dentin-like bridges. Consequently, defects in CS/Exo group had significantly higher area percentage of reparative dentin (17.46 ± 1.27%) than defects in CS group (3.05 ± 0.52%, $P = 0.014$) and CS/PBS group (10.60 ± 0.29%, $P = 0.037$), and was comparable to that in CS/MTA group (21.77 ± 2.67%; $P = 0.674$) (Fig. 4F).

The enhanced formation of reparative dentin bridges observed in the CS/Exo group was accompanied by increased expression of DSPP and DMP1 (Fig. 4D–E). Notably, CS/Exo group had significantly higher expression of DSPP (17.17 ± 1.45%) than CS group (2.41 ± 0.77%; $P < 0.001$) and CS/PBS group (2.96 ± 0.63%; $P < 0.001$) and was comparable to that in CS/MTA group (18.21 ± 0.46%; $P = 0.716$) (Fig. 4G). Similarly, CS/Exo group had significantly higher expression of DMP1 (15.77 ± 1.67%) than CS group (3.36 ± 1.03%; $P = 0.003$) and CS/PBS group (3.88 ± 0.91%; $P = 0.002$) and was comparable to that in CS/MTA group (16.53 ± 0.63%; $P = 0.995$) (Fig. 4H).

3.4. MSC exosomes induce pulp-dentin-like tissue formation in a subcutaneously implanted human premolar model

Next, we evaluated the efficacy of MSC exosomes for pulp-dentin formation in the root canal of endodontically-treated human premolars. In this model, collagen gel mixed with MSC exosomes (CG/Exo) or PBS (CG/PBS) were injected into the root canal of human premolar root fragments, and the assembled constructs were subcutaneously implanted into the mouse dorsum (Fig. 5A). Untreated human premolars were used as naive control. At 5 weeks, all the constructs (6 out of 6) in CG/Exo group showed robust recellularization in the root canal space with newly formed dentin-like tissues along the canal walls (Fig. 5B–D). In contrast, all of the constructs (6 out of 6) in the CG/PBS group had minimal tissue formation (Fig. 5B–D). Consequently, CG/Exo group had significantly higher percentage of reparative dentin tissues (4.92 ± 0.73%) than CS/PBS group (0.89 ± 0.40%; $P = 0.005$) but lower than that in the naive human dental pulp (9.37 ± 1.79%; $P = 0.003$) (Fig. 5G). The newly formed tissues in the CG/Exo group expressed both DSPP and DMP1, predominantly distributed at the interface of recellularized pulp-like tissue and dentin-like tissue (Fig. 5E–F). Semi-quantification of the positively stained area further showed that CG/Exo group had significantly higher expression of DSPP than the CG/PBS group (2.98 ± 0.50% vs 0.52 ± 0.17%; $P = 0.002$) but was lower than that in the naive control (5.12 ± 0.69% $P = 0.021$) (Fig. 5H). Similarly, CG/Exo group had significantly higher expression of DMP1 than the CG/PBS group (3.26 ± 0.25% vs 0.47 ± 0.16%; $P < 0.001$) but lower than that in the naive control (6.77 ± 1.48% $P = 0.029$) (Fig. 5I). In addition, the cell morphology and matrix organization were seemingly different between the exosome-recellularized tissues and the human dental pulp.

4. Discussion

In this study, we demonstrated that human MSC exosomes work through a multi-faceted mechanism of enhancing DPC migration, proliferation, odontogenic differentiation and mineralization, which are cellular processes critical for dental pulp regeneration. Consistent with these therapeutic effects of MSC exosomes on DPC functions, we observed enhanced pulp-dentin tissue formation with exosome treatment in both rat pulp defect model and subcutaneously implanted human premolar model.

Unlike previous studies that employed EVs derived from dental tissue sources such as dental pulp and exfoliated deciduous teeth for dental pulp regeneration [23–25], we showed here that native MSC exosomes derived from an immortalized E1-MYC 16.3 MSC line [26] could promote pulp-dentin regeneration, without the need for isolation of primary dental tissues. These cells grow faster and have increased telomerase activity while retaining the parental karyotype, thus providing an unlimited supply of cells for a scalable production of exosomes in a consistent and reproducible manner required for clinical translation [26]. Although not derived from a dental/oral tissue origin, these MSC exosomes exhibited prolific effects on enhancing DPC functions critical for dental pulp regeneration.

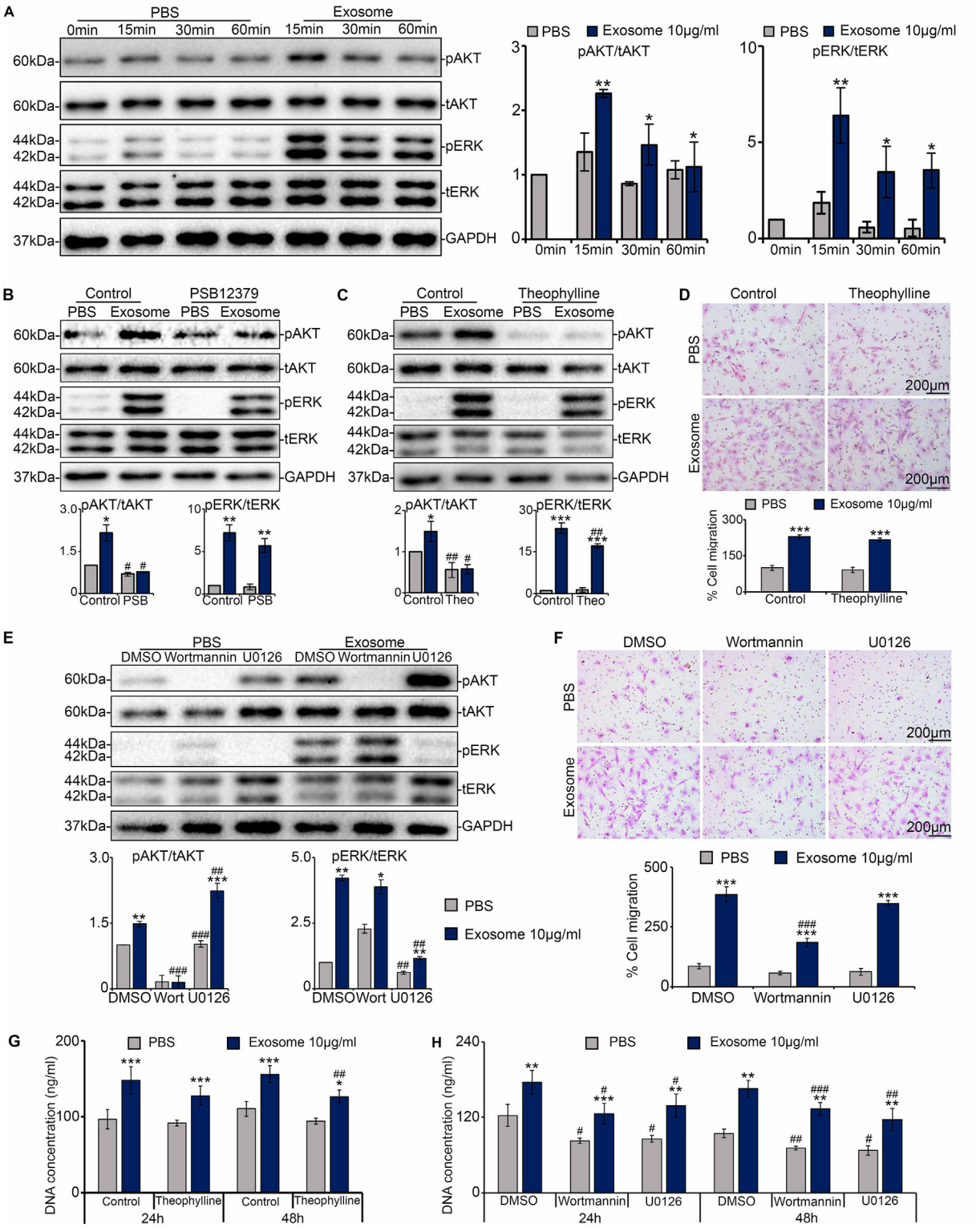

Fig. 3. MSC exosomes modulate DPC proliferation and migration partly through adenosine receptor activation of AKT and ERK signalling. (A) Western blotting and semi-quantification showed rapid AKT and ERK phosphorylation in DPC cells following exposure to 10 μg/ml exosomes. (B) Western blotting and semi-quantitative analysis of CD73 inhibition by PSB12379 (PSB) showed suppression of exosome-mediated phosphorylation of AKT, and ERK at a lesser extent. (C) Western blotting and semi-quantitative analysis of theophylline inhibition of adenosine receptors showed suppression of AKT and ERK phosphorylation. (E) Addition of wortmannin (wort) and U0126 respectively abrogated exosome-mediated AKT and ERK phosphorylation. (D, F) Transwell migration assay and (G-H) DNA assay showed that exosome treatment increased DPC migration and proliferation, but these effects were suppressed by (D, G) theophylline inhibition of the adenosine receptors and (F, H) AKT and ERK inhibition with wortmannin and U0126. Representative results of 3 independent experiments. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to PBS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to control or DMSO group ($n = 3$).

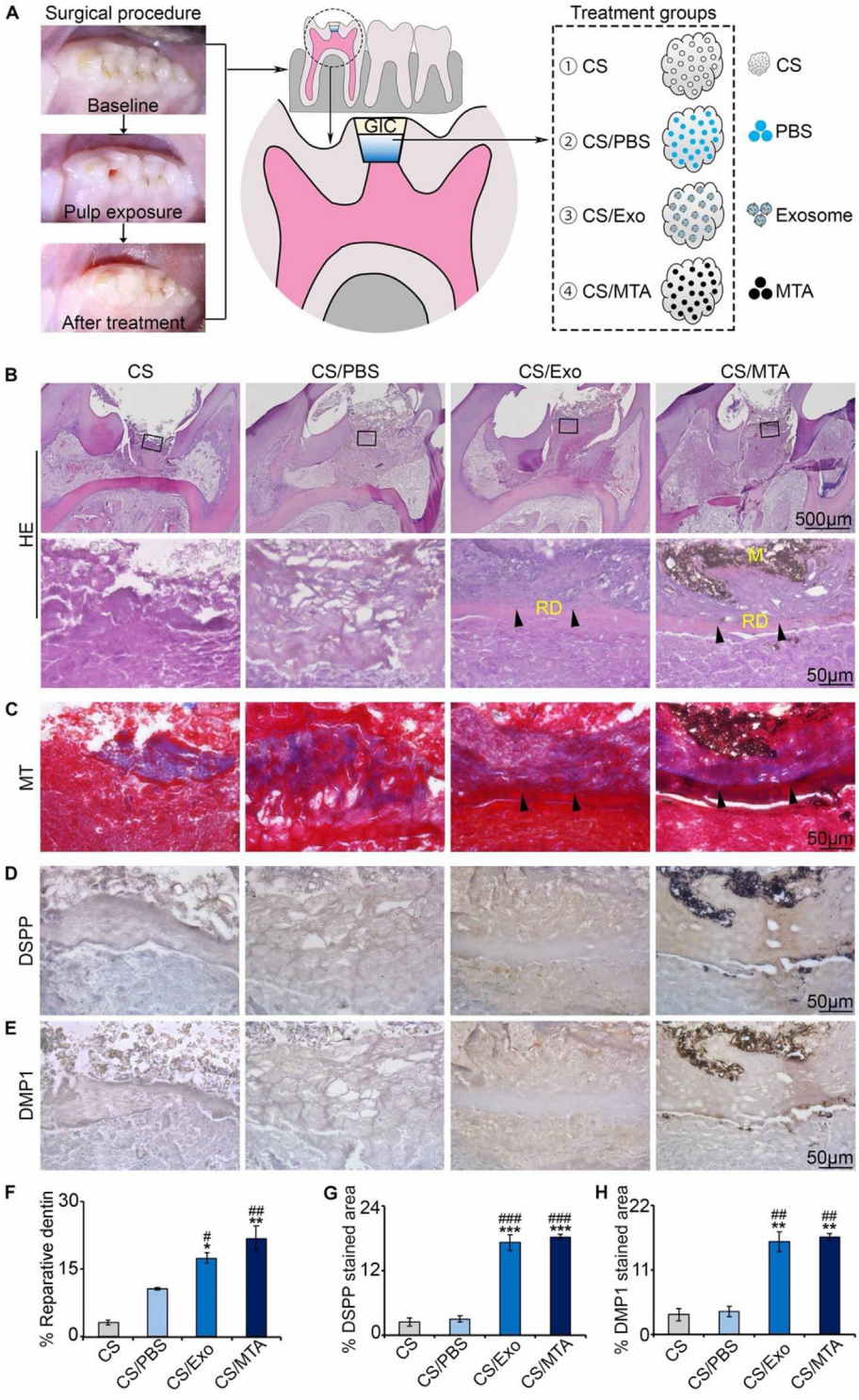

Fig. 4. MSC exosomes promote dentin-like tissue formation and enhance odontogenesis in a rat pulp defect model. (A) Animal model and experimental design. Dental pulp defect (1.5 mm in diameter and 1.5 mm in depth) was created on the occlusal surface of maxillary first molars. Following pulp exposure and hemostasis, the animals were randomly assigned to four groups (n = 8 defects/group): defects treated with collagen sponge (CS), defects treated with collagen sponge soaked with PBS (CS/PBS), defects treated with collagen sponge containing exosomes (CS/Exo), and defects treated with collagen sponge coated with mineral trioxide aggregate (CS/MTA). All defects were sealed with glass-ionomer cement (GIC). Animals were euthanized at 4 weeks for analyses. (B) Haematoxylin and eosin (HE) staining. Representative images (n = 8). Scale bars: 500 μm or 50 μm. Black box indicates the region for the magnified view. Arrowheads indicate the formation of reparative dentin bridges. RD: reparative dentin, M: MTA. (C) Masson's Trichrome (MT) staining. Representative images (n = 8). Scale bar: 50 μm. Arrowheads indicate the formation of reparative dentin bridges. (D-E) Immunohistochemical staining of (D) DSPP and (E) DMP1 showed positively stained areas surrounding the reparative dentin bridges in CS/Exo and CS/MTA groups. Representative images (n = 8). Scale bar: 50 μm. (F) Percentage area of reparative dentin. (G-H) Percentage area deposition of (G) DSPP and (H) DMP1. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to CS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to CS/PBS.

Using DPC cultures, we showed that MSC exosomes could enhance DPC migration, proliferation and odontogenic differentiation and mineralization. Notably, exosome-mediated enhancements in migration and proliferation could be observed within 24 h, and in odontogenic differentiation and mineralization as early as 7 days. Based on the temporal profile of events, such rapid cellular responses are likely to be elicited through receptor-mediated activation of the survival kinases such as AKT and ERK. Indeed, MSC exosomes could induce rapid phosphorylation of AKT and ERK as early as 15 min.

We had previously reported that MSC exosomes express CD73, a surface ecto-5′-nucleotidase (NT5E) that can hydrolyze AMP to adenosines to activate AKT and ERK signaling through interaction with adenosine receptors [40]. Using PSB12379 (a CD73 inhibitor) and theophylline (a non-selective inhibitor of adenosine receptor), we determined that exosome-mediated AKT and ERK phosphorylation in DPCs was mediated by exosomal CD73/NT5E activity. We further demonstrated that MSC exosomes enhanced DPC migration, proliferation, and odontogenic differentiation, at least in part, via CD73-mediated adenosine receptor acti-

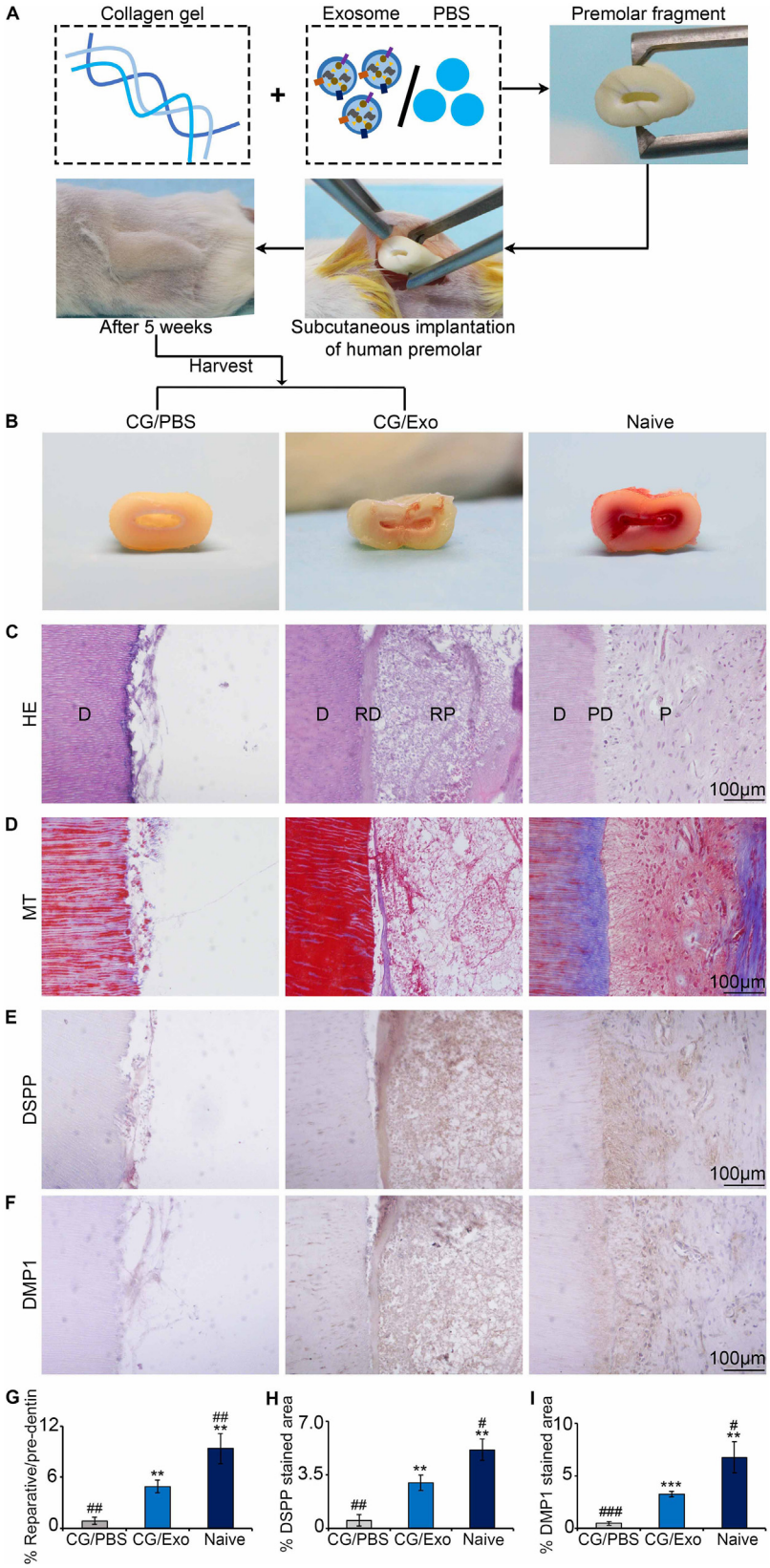

Fig. 5. MSC exosomes induce pulp-dentin-like tissue formation in subcutaneously implanted human premolar model. (A) Animal model and experimental design. Collagen gels combined with exosomes (CG/Exo) or PBS (CG/PBS) were injected into human premolar root fragments and subcutaneously implanted into the dorsum of SCID mice for 5 weeks. (B) Gross appearance. (C) Haematoxylin and eosin (HE) staining and (D) Masson's Trichrome (MT) staining. Scale bar: 100 μm. D: dentin, RD: reparative dentin, RP: regenerated pulp-like tissues, PD: pre-dentin, P: pulp. (E-F) Immunohistochemical staining of (E) DSPP and (F) DMP1 showed positively stained areas in the recellularized dentin-like tissues in CG/Exo group. Representative images ($n = 6$). Scale bar: 100 μm. (G) Percentage area of reparative dentin or pre-dentin. (H-I) Percentage area deposition of (H) DSPP and (I) DMP1. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to CG/PBS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to CG/Exo.

vation of AKT and ERK phosphorylation. However, it is noteworthy that exosome-mediated AKT and ERK phosphorylation was not completely abolished by PSB12379 or theophylline, and there could be exosome cargo proteins other than CD73 that could mediate AKT and/or ERK phosphorylation. Indeed, the MSC exosome proteome is highly complex [41] and contain several factors such as the fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin growth factor (IGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF) that could activate the AKT and/or ERK signaling in DPCs [42–44]. For instance, VEGF and IGF-1 could mediate DPC proliferation, migration, osteogenesis and angiogenesis through activation of AKT signaling [43]. Nevertheless, our findings demonstrated that exosomal CD73/NT5E-mediated adenosine activation of AKT and ERK signaling were at least partially responsible for the enhancements in DPC migration, proliferation and odontogenic differentiation and mineralization. These observations were consistent with our previous studies that MSC exosomes enhanced cell migration and proliferation of chondrocytes [32] and periodontal ligament cells [13] through CD73/NT5E-mediated adenosine activation of AKT and ERK signalling. More recently, MSC exosomes have also been reported to mediate M2-like macrophage polarization through CD73/NT5E activity [45]. These findings suggest that MSC exosomes can exert their effects without cellular uptake which is consistent with the recent reports that cellular uptake of EVs is generally inefficient [46,47].

Consistent with the therapeutic effects of MSC exosomes on DPC functions, we observed that MSC exosomes enhanced pulp-dentin regeneration in our animal models. Using a rat pulp defect model, we showed that delivery of MSC exosomes in collagen sponge (CS/Exo) increased the expression of dentin matrix proteins (DSPP and DMP1) and promoted the formation of reparative dentin and bridge-like structures that were comparable to that of MTA as the positive control, following 4 weeks of treatment.

Pulp-capping is performed in dental practice to maintain the viability and functions of the dental pulp, following injury [48]. MTA is one of the routinely used pulp-capping materials but its clinical application can be hampered by some drawbacks such as high cost, long setting time, poor handling and tooth discoloration [49]. The slow degradation of mineral-based cements may also affect tissue ingrowth and dentin formation [21]. Having demonstrated comparable efficacy of MSC exosomes as the MTA for pulp-dentin regeneration in our rat pulp defect model, we propose that MSC exosomes could be incorporated into or combined with the existing pulp-capping materials to promote pulp-dentin regeneration.

The mechanical debridement performed during endodontic treatment removes dentin from root walls making the tooth more prone to fracture [50]. Moreover, the root is often sealed with inert materials which do not promote dentin deposition or further root formation [51]. In this study, we demonstrated that administering MSC exosomes in collagen gel (CG/Exo) helped recellularization of pulp-dentin tissues in endodontically-treated human premolar, with significant expression of DSPP and DMP1 following 5 weeks of subcutaneous implantation, albeit lower in expression compared to that in naive human dental pulp tissue. Cell morphology and matrix organization between the exosome-recellularized tissues and the human dental pulp were also seemingly different, and this could be explained by the different cell types residing in the mouse subcutaneous space and in the dental pulp/periapical tissues. Nevertheless, our findings suggest the potential use of MSC exosomes for pulp-dentin regeneration and revitalization.

To apply exosomes as therapeutic agent during endodontic procedures, they should be amenable to different methods of delivery, and compatible for direct use with current clinical materials in different clinical scenarios. Collagen sponges and gels are commercially available and widely used as scaffolds for delivering stem cells and/or growth factors for dental pulp regeneration [51]. In this study, we demonstrated that MSC exosomes are amenable for delivery in collagen sponge for implantation and in collagen gel for injection, in animal models of dental pulp defect and endodontically-treated human premolar. There was also no apparent loss of activity of MSC exosomes when applied in conjunction with GIC used to seal the pulp defect.

Through our animal studies, we have also demonstrated that a single dose of MSC exosomes delivered in collagen sponge or gel promoted pulp-dentin regeneration *in vivo*. This observation is consistent with our previous animal studies that a single dose of MSC exosomes delivered in collagen sponge was able to enhance periodontal regeneration by 4 weeks [13] and sustain repair and regeneration of cranial bone defects to 8 weeks [52]. This finding that a transient exposure of MSC exosomes could trigger a sustained regenerative process is in concordance with our long-standing hypothesis that MSC exosomes work rapidly to restore homeostasis and initiate endogenous tissue repair and regeneration [53].

Cell migration/homing approach of utilizing growth factors/cytokines has been previously proposed to promote dental pulp regeneration [54]. However, the use of single or a combination of a few factors may not be sufficient to coordinate the complex process of pulp-dentin regeneration that involves multiple factors and several cell types. As such, MSC exosomes with a rich diverse proteomic and RNA cargo are ideal for this synergism of multiple factors and cellular activities [41,55]. These cargo components are functionally complex and have been implicated in many diverse and cellular biochemical processes such as cell-cell communication, metabolism and tissue repair and regeneration [53].

There are some limitations to our study. We have only evaluated a single dose of MSC exosomes in our study. Further optimization of the exosome dose and scaffold delivery will be required to improve dental pulp regeneration. A longer study may be required to achieve structural restoration and organization of the dental pulp with mature tubular structure of tertiary dentin and thickening of dentin walls. Further investigation in a larger, clinically relevant animal model would also be required for clinical translation [56].

5. Conclusion

In summary, we have demonstrated that human MSC exosomes are capable of mounting a multi-faceted response of enhancing DPC functions such as migration, proliferation and odontogenic differentiation that culminated in enhanced pulp-dentin regeneration in both models of rat dental pulp defect and subcutaneously implanted human premolar. We further showed that MSC exosomes enhanced DPC functions through exosomal CD73/NT5E-mediated adenosine activation of AKT/ERK signaling pathways. Our findings provide the basis for future development of human MSC exosomes as a cell-free therapeutic for pulp-dentin regeneration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sai Kiang Lim reports a relationship with Paracrine Therapeutics Pte Ltd that includes: equity or stocks.

Data availability

Data will be made available on request.

Disclosure

SKL holds founder shares in Paracrine Therapeutics Pte Ltd. Other authors report that they have no conflicts of interest in the authorship and publication of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbiosy.2023.100078.

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