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RAPID COMMUNICATION

# Mesenchymal stromal cell-derived small extracellular vesicles modulate macrophage polarization and enhance angio-osteogenesis to promote bone healing

Mesenchymal stem/stromal cells (MSCs) have demonstrated therapeutic efficacy for bone regeneration in animal and clinical studies. Although MSCs were initially thought to differentiate to various cell types to replace the injured/diseased tissue, it is now accepted that these cells secrete factors to promote tissue repair.[1] Among these factors, small extracellular vesicles (sEVs) of size 50–200 nm, which include the exosomes, have been identified as the principal agent mediating the wide-ranging therapeutic efficacy of MSCs.[2] Several studies have also reported the therapeutic effects of MSC-sEVs to enhance bone repair in animal models, as recently reviewed.[3] However, the cellular processes and mechanisms mediated by MSC-sEVs in bone regeneration remain to be fully elucidated.

In this study, we employed a rat calvaria defect model to investigate the cellular processes activated by MSC-sEVs during bone repair (Supplementary Methods). Briefly, MSC-sEVs were prepared as previously described,[4] and characterized in accordance with the MISEV2018 guidelines.[5] Collagen sponges with 100 μg MSC-sEVs in 100 μl PBS (CS/sEVs) or equivalent volume of phosphate-buffered saline (PBS, CS/Control) were implanted to 8-mm diameter critical-size calvaria defects surgically created in 32 rats. Another five rats served as age-matched naive control. At 1 and 8 weeks, samples were harvested for analyses using micro-computed tomography (micro-CT), histology and immunohistochemistry.

Micro-CT analysis revealed minimal bone formation in both groups at 1 week (Fig. S1). Despite limited bone formation, CS/sEVs group showed better cell infiltration and matrix deposition than the CS/Control group (Fig. 1A). Differences were more evident at 8 weeks, with CS/sEVs group showing new bone that completely bridged the defect, as opposed to CS/Control group having limited new bone, mostly confined to the defect periphery (Fig. 1A). Notably, CS/sEVs displayed significantly higher BV/TV, Tb.Th and Tb.N, but lower Tb.Sp than the CS/Control group and was comparable to that of the naive control (Fig. S1). Consequently, CS/sEVs group had an overall micro-CT score of $3.9 \pm 0.2$ that was significantly higher than CS/Control group with $2.5 \pm 0.8$ and was comparable to that of the naive control with $4 \pm 0.0$ (Fig. S1). At 8 weeks, CS/sEVs group displayed histological improvements in new bone formation, marrow changes and bone cortex remodelling that culminated in a significantly better score of $5.4 \pm 1.0$ over the CS/Control group with $2.6 \pm 1.8$ (Fig. 1B). Compared to the naive control, the defect repair in CS/sEVs group was near completion as evidenced by a slightly lower but statistically not different scores in the histological parameters measured.

Further analyses by immunohistochemistry revealed significantly higher percentage areal deposition of osteocalcin (OCN) in the CS/sEVs group than the CS/Control group, that persisted to 8 weeks (Fig. 1C). By 8 weeks, CS/sEVs group had ~2.5-fold higher areal deposition of OCN than CS/Control group (Fig. 1C). On the other hand, CS/sEVs group had ~2-fold higher number of $CD31^+$ microvessels than CS/Control group at 1 week (Fig. 1D). However, the number of $CD31^+$ microvessels declined at 8 weeks in both groups with no significant difference (Fig. 1D). Additionally, CS/sEVs group showed a significantly higher number of $PCNA^+$ proliferative cells than the CS/Control group at 1 week. The number of $PCNA^+$ cells declined at 8 weeks in both groups with no

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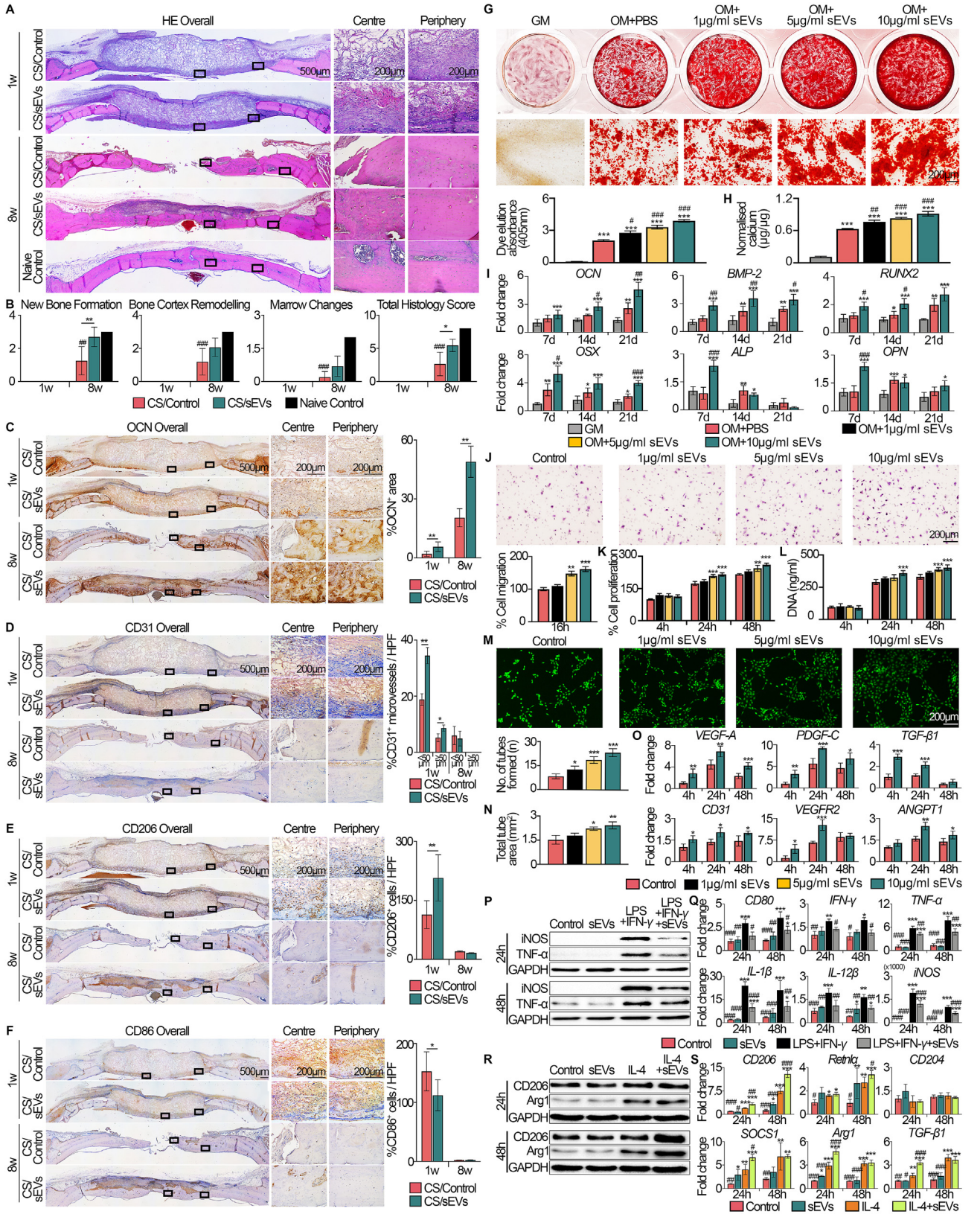


**Figure 1** MSC-sEVs promote bone regeneration through increased angiogenesis and osteogenesis, and enhanced M2 over M1 macrophage infiltration. **(A)** Histological and histomorphometric analyses of calvarial bone regeneration at 1 and 8 weeks by hematoxylin and eosin (HE) staining and modified histological scoring **(B)** that assessed parameters including new bone formation, marrow changes and cortex remodeling. Data are presented as mean ± SD ($n = 8$), $^{*}P < 0.05$, $^{**}P < 0.01$ compared to CS/Control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to naive control group. Immunohistochemical staining and quantitative analysis of $OCN^{+}$ stained

significant difference (Fig. S2A). Conversely, the number of $CCP3^+$ apoptotic cells were similar in both CS/sEVs and CS/Control groups at 1 week but declined at 8 weeks in both groups (Fig. S2B). As immune response is integral to tissue repair upon injury, we examined if there was a difference in macrophage infiltration into the defect site during bone regeneration. As early as 1 week, we observed an abundance of $CD206^+$ M2 macrophages, with CS/sEVs group having a significantly higher number of $CD206^+$ cells than the CS/Control group (Fig. 1E). Thereafter, the number of $CD206^+$ cells declined in both groups with no significant difference (Fig. 1E). In contrast to M2 macrophages, the number of $CD86^+$ M1 macrophages in the CS/sEVs group was significantly lower than that in CS/Control group at 1 week (Fig. 1F). By 8 weeks, the number of $CD86^+$ cells declined in both groups with no significant difference (Fig. 1F). These observations were further supported by the evaluation of M1 macrophage-associated pro-inflammatory cytokines, namely IL-1$\beta$ and TNF-$\alpha$. At 1 week, CS/sEVs group relative to CS/Control group had significantly lower numbers of IL-$1\beta^+$ and TNF-$\alpha^+$ cells. At 8 weeks, the number of IL-$1\beta^+$ and TNF-$\alpha^+$ cells declined in both groups with no statistically significant difference (Fig. S3A, B). Collectively, our findings demonstrated that MSC-sEV-mediated bone healing is largely characterized by increased cellular proliferation and infiltration, improved vascularization, and mineralization, and enhanced M2 over M1 macrophage infiltration with reduced inflammation.

To gain insights into the cellular processes activated by MSC-sEVs during bone repair, cell culture studies utilizing MSCs, endothelial cells and macrophages were performed (Supplementary Methods). Consistent with our animal results that observed enhanced new bone formation, we found that MSC-sEVs significantly enhanced osteogenic differentiation of MSCs in a dose-dependent manner with 10 μg/ml MSC-sEVs demonstrating the highest levels of eluted dye and calcium deposition (Fig. 1G, H). This enhancement in osteogenic differentiation by MSC-sEVs was also supported by our gene expression analysis showing upregulation of osteogenic genes such as *OCN*, *BMP-2*, *RUNX2*, *OSX*, *ALP* and *OPN* (Fig. 1I). Similarly, we observed that MSC-sEVs enhanced endothelial cell migration, proliferation, and tube formation in a dose-dependent manner, with 10 μg/ml MSC-sEVs having the most potent effects (Fig. 1J–N). Our gene expression analysis also showed that MSC-sEVs elevated several angiogenic genes including *VEGF-A*, *PDGF-C*, *TGF-β1*, *CD31*, *VEGFR2* and *ANGPT1* (Fig. 1O). These findings suggest that the enhanced vascularization and mineralization observed during bone repair in our animal model could be attributed to MSC-sEV-enhanced angiogenesis and osteogenesis.

Next, we tested the effects of MSC-sEVs on macrophage polarization. Naive M0 macrophages were treated with LPS and IFN-γ (M1) or IL-4 (M2) to polarize to M1 or M2 macrophages, respectively. We found that MSC-sEVs suppressed the expression of M1 macrophage markers, iNOS and TNF-α, and the associated genes including *CD80*, *IFN-γ*, *TNF-α*, *IL-1β*, *IL-12β* and *iNOS* in LPS+IFN-γ+sEVs group, compared to LPS+IFN-γ group (Fig. 1P, Q). On contrary to the suppressive effects of MSC-sEVs on (LPS+IFN-γ)-mediated M1 polarization, MSC-sEVs treatment was able to enhance (IL-4)-mediated M2 polarization as evidenced by the increased expression of M2 macrophage markers, CD206 and Arg1, and associated genes, including *CD206*, *Retnlα*, *SOCS1*, *Arg1* and *TGF-β1* (Fig. 1R, S). Despite having effects on M1 or M2 polarized macrophages, MSC-sEVs had little effects on the expression of M1 or M2 macrophage-associated markers by the naive macrophages (Fig. 1P–S). These results suggest that the preferential M2 over M1 macrophage infiltration observed during bone repair in our animal model could be attributed to the enhanced M2 over M1 macrophage polarization mediated by MSC-sEVs.

In summary, we observed that MSC-sEV-mediated bone healing was characterized by increased cellular proliferation and infiltration, enhanced vascularization and mineralization, and reduced inflammation with a regenerative macrophage phenotype. By means of cell

areas **(C)** and $CD31^+$ microvessels **(D)**. Data are presented as mean ± SD ($n = 8$). $^*P < 0.05$, $^{**}P < 0.01$ compared to CS/Control. Immunohistochemical staining and quantitative analysis of $CD206^+$ **(E)** and $CD86^+$ cells **(F)**. Data are presented as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$ compared to CS/Control. MSC-sEVs enhanced osteogenic differentiation of MSCs **(G–I)**. MSCs were cultured in osteogenic medium (OM) supplemented with 1, 5 and 10 μg/ml of sEVs or vehicle (PBS) for 21 days. MSCs cultured in growth medium (GM) served as a control. **(G)** Alizarin red S staining with quantification of eluted dye and calcium assay **(H)** showed dose-dependent effect of MSC-sEVs on osteogenic differentiation of MSCs. **(I)** RT-qPCR analysis showed upregulation of genes associated with osteogenesis (*OCN*, *BMP-2*, *RUNX2*, *OSX*, *ALP*, *OPN* ). Data are presented as mean ± SD ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to growth medium control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to OM+PBS group. MSC-sEVs also promoted migration, proliferation and tube formation of endothelial cells (EA.hy926) **(J–O)**. **(J)** Transwell migration assay showed dose-dependent effect of MSC-sEVs on endothelial cell migration. **(K)** MTS cell metabolic activity assay and DNA assay **(L)** showed potent dose and time-dependent effects of MSC-sEVs on endothelial cell metabolic activity and proliferation. Tube formation assay measuring the number of tubes **(M)** and total tube area **(N)** showed dose-dependent effects of MSC-sEVs on angiogenesis. **(O)** RT-qPCR analysis showed upregulation of genes associated with angiogenesis (*VEGF-A*, *PDGF-C*, *TGF-β1*, *CD31*, *VEGFR2* and *ANGPT1*). Data are presented as mean ± SD ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control. MSC-sEVs enhanced M2 over M1 polarization of rat alveolar macrophages (NR8383) **(P–S)**. **(P)** Western blot analysis of iNOS, TNF-α and GAPDH. **(Q)** RT-qPCR analysis of genes associated with M1 macrophages (*CD80*, *IFN-γ*, *TNF-α*, *IL-1β*, *IL-12β*, and *iNOS* ). **(R)** Western blot analysis of CD206, Arg1 and GAPDH. **(S)** RT-qPCR analysis of genes associated with M2 macrophages (*CD206*, *Retnlα*, *CD204*, *SOCS1*, *Arg1* and *TGF-β1*). Data are presented as mean ± SD ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to LPS+IFN-γ group or IL-4 group.

culture studies, we could attribute some of the cellular activities mediated by MSC-sEVs during bone repair to increased osteogenic mineralization of MSCs, enhanced migration and tube formation of endothelial cells, as well as anti-inflammatory M2 over pro-inflammatory M1 polarization of the macrophages.

## Author contributions

Investigation, formal analysis, data curation, visualisation and writing — original draft: SJC,: CWY, KYWT, JRJC, YAC, SZ. Writing — reviewing and editing: RCWW, AATL. SKL: Funding acquisition, writing — reviewing and editing. WST: Conceptualization, methodology, validation, data curation, supervision, funding acquisition, writing — reviewing and editing.

## Conflict of interests

SKL holds founder shares in Paracrine Therapeutics Pte Ltd. All other authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.11.012.

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