Human iPSC-Derived Extracellular Vesicles (hiPSC-EVs) Promote Osteogenic Differentiation

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INTRODUCTION: Stem cell regenerative medicine has been applied in clinical treatment, including the treatment of severe bone defects, but it still has limitations such as high manufacturing cost, limited number of cells, and cell transformation risk. The efficacy of stem cell therapy may be from the cell itself or/and cell' s secretomes. Compared to the stem cells, cell' s secretomes are safer and lower in manufacturing cost because they do not contain cells themselves, which overcome the limitations of stem cell therapy. Secretomes contain growth factors, small molecule peptides, and extracellular vesicles (EV). Currently, many studies focus on secretomes from mesenchymal stem cells (MSC), but the studies of induced pluripotent stem cells (iPSC) secretome are seldom reported**.** iPSC should be more proliferative than MSC, more diverse in cell differentiation, and more specific cell sources can produce more stable EV, which may be more suitable for bone regenerative medicine. Therefore, this study intends to investigate the effect of human iPSC-EV on the osteogenic differentiation of murine marrow mesenchymal stem cell (D1) and osteoblast (MC3T3-E1).

METHODS: Murine bone marrow mesenchymal stem cells (D1 cells) and osteoblasts (MC3T3E1) were used in this study. The human iPSC line (IBMS-iPSC-01-02_feeder-free) will be used to produce EVs which were purified by ultra-high-speed centrifugation methods. To evaluate the osteogenesis, the different concentration of hiPSC-EVs were used in D1 cells and MC3T3E1 cells. The mRNA level of BMP-2, Runx-2, ALP, osteocalcin (OC) and collagen 1 were quantified using the Q-PCR. The mineralization of extracellular matrix (ECM) calcification after osteogenic induction was determined by Alizarin red S staining. The miRNA profile of hiPSC-EVs were analyzed by miRNA NGS analysis. Each experiment was performed at least 3 times. Significant differences between groups were tested using one-way ANOVA, and multiple comparisons were tested using Scheffe's method. The thresholds for significance and high significance were $P < 0.05$ and $P < 0.01$, respectively.

RESULTS: The hiPSC-EVs were identified by NTA and TEM revealed the concentration of $2.4 \pm 0.4 \times 10^{10}$ particles/ml and a particle size of 118.7±17.2nm (Fig 1A and B). Western blot showed that the sample expresses ALIX, TSG101, CD63, and CD81, and Calreticulin is not expressed (Fig 1C), which confirmed the sample is hiPSC-EV. To confirm whether hiPSCs-EV can promote osteogenesis, the MC3T3E1 cells and D1 cells were used. We evaluated the mineralization (calcium deposition) and osteogenic gene expressions. The results showed that the mineralization were increased after hiPSC-EVs treatments and with dose dependent manner in MC3T3E1 (Fig 2A). The expression of osteogenic gene, Runx2, BMP2, ALP, COL-1 and OC, were increased after hiPSC-EV treatment in MC3T3E1 cells (Fig. 2B). In D1 cells, the mineralization and osteogenic genes were also increased by hiPSC-EV treatments (Fig. 2B). The miRNA of hiPSC-EVs was analyzed by NGS. The top expression miRNAs were evaluated which miR-302b-3p had highest expression (Fig 3A). We performed GO analysis associated with the identified miRNA target genes in hiPSCs. The major target genes were associated with neuroprojection organization, extracellular exosome biogenesis, EV biogene sis, post-synaptic endosomes, axonal growth cones, nuclear import signalling, and receptor activity (Fig 3B). In KEGG analysis, the hiPSC-Exos miRNAs significantly affected mitogen-activated protein kinase (MAPK) signalling, Hippo signalling, signalling pathway regulating pluripotency of stem cells, longevity regulating pathway, PI3K-Akt, Wnt signalling, focal adhesion, cell senescence, and cell cycle (Fig 3C).

DISCUSSION: Bone is a unique tissue in the human body, capable of scarless self-renewal. However, when faced with significant trauma, disease, or other factors, its ability to self-heal can fail resulting in nonunion fractures or bone defects. There are several threptic strategies to overcome bone defect, but it still has some limitation. In this study, we found hiPSC-EVs can promote osteogenesis in D1 and MC3T3E1 cells. It increased mineralization and enhanced expression of osteogenic genes following the administration of hiPSC-EVs.

SIGNIFICANCE/CLINICAL RELEVANCE: These results suggest that hiPSC-EVs hold potenital for clinical use in promoting bone regeneration.

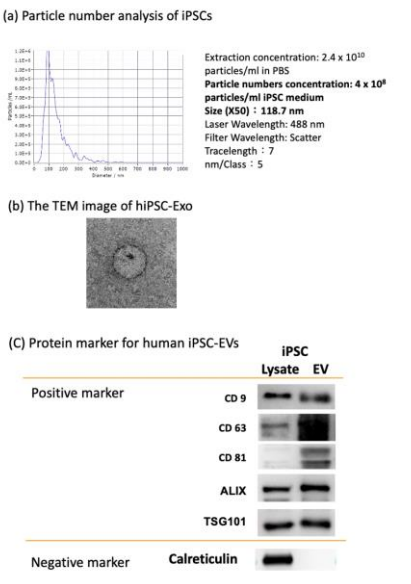

Fig.1 The mRNA expression and protein levels after inhibition of COX-2 or COX-2 silencing. (A) The mRNA expression after COX-2 inhibition (B) mineralization after COX-2 inhibition (C) Protein levels of FOXO3a and $p27^{kip1}$ (D) The mRNA expression after COX-2 silencing. (n=4)

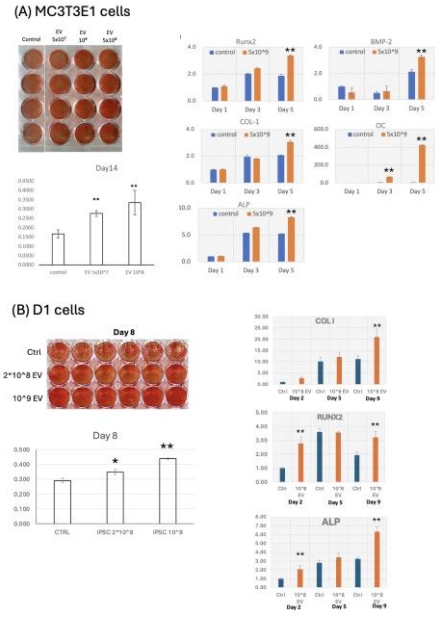

Fig. 2. The mRNA expression and protein levels after overexpression of COX-2 (A) The mRNA expression of BMP-2, Runx-2 OC and ALP. (B) The protein levels of FOXO3a, p27kip1 and Runx-2. (n=4)

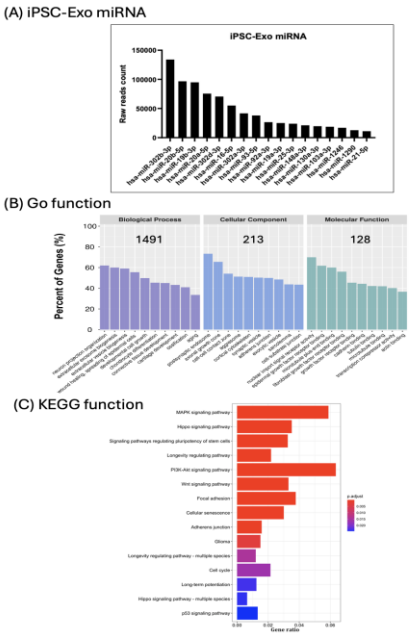

Fig. 3. COX-2 plays an important role in osteogenesis and proliferation