

# Human Mesenchymal Stem Cell-Derived Exosomes Promote the Proliferation and Melanogenesis of Primary Melanocytes by Attenuating the H<sub>2</sub>O<sub>2</sub>-Related Cytotoxicity in vitro

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**Background:** Mesenchymal stem cell-derived exosomes (MSC-Exo) have therapeutic potential. However, the impact of MSC-Exo on the survival and melanogenesis of human primary melanocytes following H<sub>2</sub>O<sub>2</sub>-induced damage has not been clarified. We therefore investigated the effects of MSC-Exo on the H<sub>2</sub>O<sub>2</sub>-affected survival of human primary melanocytes and their proliferation, apoptosis, senescence, and melanogenesis in vitro.

**Methods:** MSC-Exo were prepared from human MSCs by sequential centrifugations and characterized by Transmission Electron Microscopy, Western blot and Nanoparticle Tracking Analysis. Human primary melanocytes were isolated and treated with different concentrations of MSC-Exo, followed by exposing to H<sub>2</sub>O<sub>2</sub>. Furthermore, the impact of pretreatment with MSC-Exo on the proliferation, apoptosis, senescence and melanogenesis of melanocytes were tested by CCK-8, flow cytometry, Western blot, L-Dopa staining, tyrosinase activity and RT-qPCR.

**Results:** Pretreatment with lower doses of MSC-Exo protected human primary melanocytes from the H<sub>2</sub>O<sub>2</sub>-triggered apoptosis, while pretreatment with higher doses of MSC-Exo enhanced the H<sub>2</sub>O<sub>2</sub>-induced melanocyte apoptosis. Compared with the untreated control, pretreatment with a lower dose (1 µg/mL) of MSC-Exo enhanced the proliferation of melanocytes, abrogated the H<sub>2</sub>O<sub>2</sub>-increased p53, p21, IL-1β, IL-6 and IL-8 expression and partially rescued the H<sub>2</sub>O<sub>2</sub>-decreased L-dopa staining reaction, tyrosinase activity, MITF and TRP1 expression in melanocytes.

**Conclusion:** Our findings indicate that treatment with a low dose of MSC-Exo promotes the proliferation and melanogenesis of human primary melanocytes by ameliorating the H<sub>2</sub>O<sub>2</sub>-induced apoptosis and senescence of melanocytes. MSC-Exo may be a promising therapeutic strategy of vitiligo.

**Keywords:** mesenchymal stem cell-derived exosomes, melanocytes, apoptosis, proliferation, senescence

## Introduction

Vitiligo is one of the common skin diseases and characterized by the selective destruction of epidermal melanocytes, leading to depigmented patches in the skin or mucous membranes. Vitiligo was first documented as far back as 3500 years ago,<sup>1</sup> and currently affects about 0.5–2% of people in the world.<sup>2,3</sup> Vitiligo affects people of all Fitzpatrick skin types,<sup>4</sup> but it may be more noticeable on darker skin.<sup>5</sup> Vitiligo can involve any part of the body, particularly for the visible areas, such as the face and arms, and even spread to whole body, causing great psychological distress to patients and seriously affecting quality of life of both the patients and their family members.<sup>6,7</sup>

In recent years, scientific researchers have made significant progress in understanding the pathogenesis of vitiligo and helped in developing better therapeutic strategies.<sup>1,8</sup> Oxidative stress and autoimmunity-induced melanogenesis impairment and death of melanocyte have been considered to be the two key factors that contribute to the pathogenesis of vitiligo.<sup>2</sup> It is currently believed that oxidative stress plays a pivotal role in initiating the autoimmune response associated with vitiligo<sup>9</sup> and IFN- $\gamma$ -CXCL9/CXCL10-CXCR3 Axis leads to melanocyte destruction in vitiligo.<sup>10–12</sup> Furthermore, premature senescence of melanocytes is also crucial for the development and progression of vitiligo.<sup>13,14</sup> Although a variety of treatments are available, including phototherapy,<sup>15</sup> topical medications,<sup>2</sup> systemic drugs,<sup>16</sup> surgery<sup>17</sup> and targeted therapies,<sup>8</sup> the efficacy of these treatments still remains unsatisfactory.<sup>2,18</sup> Nearly 40% of vitiligo patients relapse, due to lesional CD8<sup>+</sup> resident memory T cells (T<sub>RM</sub>),<sup>19</sup> within one year after discontinuation of treatment.<sup>20</sup> Therefore, there is an urgent need to explore more new treatments.

Mesenchymal stem cells (MSCs) have unique characteristics with immunomodulatory, pro-survival, pro-angiogenic, anti-apoptotic, and anti-inflammatory activities,<sup>21</sup> and have potential to be used as a novel therapeutic strategy for the intervention of a variety of diseases.<sup>22</sup> Mechanistically, MSCs can secrete many soluble factors and release extracellular vesicles (EVs).<sup>23–25</sup> Exosome is one of the main classes of EVs. Interestingly, the functions of MSC-derived exosomes (MSC-Exo) are similar to MSCs, whereas more stable, less immunogenic and with little risk of tumorigenesis.<sup>26</sup> Recent studies have shown that MSC-Exo can protect and promote the regeneration of a variety of cells and tissues.<sup>27–29</sup> MSC-Exo can also have immunomodulatory, anti-apoptotic, antioxidant, and anti-senescence activities.<sup>30–33</sup> In the field of dermatology, MSC-Exo have been shown to be a promising novel therapeutic option for the treatment of wound healing, atopic dermatitis, hair loss and aging.<sup>34</sup> However, the impact of MSC-Exo on the survival and function of melanocytes is not clarified.

In this study, we tested the effects of pretreatment with MSC-Exo on the H<sub>2</sub>O<sub>2</sub>-affected survival of human primary melanocytes and their proliferation, apoptosis, senescence, and melanogenesis *in vitro*. Our findings indicated that pretreatment with MSC-Exo protected from the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against human primary melanocytes by promoting their survival and melanogenesis and inhibiting their apoptosis and senescence, suggesting that MSC-Exo may be promising for the treatment of vitiligo.

## Materials and Methods

### Preparation of Human Primary Melanocytes

Written informed consent was obtained from all donors, and the experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (approval number: [2023]215). Human foreskin tissues were obtained from individual male subjects (n=30, 20–28 years old), who underwent circumcision and their primary melanocytes were isolated. Individual specimens were sequentially washed with 75% ethanol and PBS solution containing 1% penicillin-streptomycin. The specimens were digested with 0.25% Dispase II solution at 4°C for 16–20 hours. The epidermis was treated with 0.25% trypsin for 15 minutes and cultured in melanocyte medium (iCell, Shanghai, China) supplemented with 0.5% foetal bovine serum (FBS) (iCell) and 1% human melanocyte growth supplement (iCell) at 37°C for 14 days. The morphology of melanocytes was observed under a microscope, and the melanocytes were identified by L-Dopa staining (2 mg/mL levodopa in PBS, Sigma, MO, USA) at a temperature of 37°C for 1 hour.

### Isolation and Characterization of Exosomes

Human umbilical cord mesenchymal stem cells (hUC-MSCs) were kindly provided by Prof. Wenbin Ma at the School of Life Sciences, Sun Yat-sen University. The hUC-MSCs at passage of 1–2 were cultured in DMEM (Gibco, NY, USA) supplemented with 10% FBS at 37°C for 14 days. Subsequently, the cells were cultured in FBS-free hUC-MSCs exosome secretion-promoting medium Ultra CULTURE<sup>TM</sup> (Lonza, Basel, Switzerland) for 48 hours, followed by collecting their culture supernatants for the isolation of exosomes by continual centrifugations.<sup>35</sup> In brief, the supernatants were sequentially centrifuged at 4°C at 300×g for 10 min, at 2000×g for 10 min, at 10,000×g for 30 min and 100,000×g for 90 min. The pellets were resuspended in PBS and centrifuged at 100,000×g for 90 min, followed by re-suspending in PBS. The MSC-Exo were quantified using the BCA kit (Thermo Fisher, MA, USA) at a wave length of 562 nm and characterized by Transmission Electron Microscopy (TEM) (Hitachi,

Tokyo, Japan) and their protein markers of CD9, CD63, and CD81 were analysed by Western blot. The concentrations and size distribution of MSC-Exo were determined using Nanoparticle Tracking Analysis (NTA) (Malvern Panalytical, WILT, UK).

## Cell Treatment

Previous studies have shown that treatment with H<sub>2</sub>O<sub>2</sub> can induce the senescence, caspase-3-dependent apoptosis and necrosis, dependent on the concentrations of H<sub>2</sub>O<sub>2</sub>.<sup>36,37</sup> Based on previous studies<sup>38,39</sup> and our preliminary experiments, treatment with 750 µmol/L of H<sub>2</sub>O<sub>2</sub> reduced the viability and proliferation of human primary melanocytes, but did not cause excessive cell necrosis. Accordingly, human primary melanocytes (1x10<sup>5</sup> cells/well) were treated with, or without, 750 µmol/L of H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich, MO, USA) for 24 hours. In addition, the melanocytes were pre-cultured with 1–40 µg/mL of MSC-Exo for 24 hours and exposed to 750 µmol/L of H<sub>2</sub>O<sub>2</sub> for another 24 hours.

## Western Blot (WB)

The different groups of cells were harvested and lysed, followed by centrifugation. After determining the protein concentrations, the cell lysate samples (40 µg/lane) were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% fat-free dry milk and incubated with primary antibodies overnight at 4°C. The primary antibodies included anti-caspase-3 (Cell Signaling Technology, CST, MA, USA), anti-microphthalmia-associated transcription factor (MITF) (CST), anti-tyrosinase (TYR) (Abcam, Cambridge, UK) and anti-tyrosinase related protein 1 (TRP1) (Abcam) as well as anti-β-Actin (Abclonal, Wuhan, China) and anti-GAPDH (Abmart, Shanghai, China). After being washed, the target proteins were visualized using fluorescent secondary antibodies (LI-COR, NE, USA) and chemiluminescent secondary antibodies (Ray antibody, Beijing, China and Abcam). In addition, the prepared MSC-Exo were subjected to WB analysis using primary antibodies against CD9, CD63, and CD81 (Immunoway Biotechnology, TX, USA).

## Cell Counting Kit 8 (CCK-8) Assay

The viability, proliferation, and survival of primary melanocytes were determined using the CCK-8 assay kit (Dojindo, Tokyo, Japan) according to the manufacturer's instruction. Briefly, melanocytes at 3–4 passages (1x10<sup>4</sup> cells/well) were cultured in 96-well plates for varying time periods. During the last 3-hour culture, the cells in each well were exposed to 10 µL of CCK-8 reagent. The formed formazan in each well was quantified by measuring the absorbance at 450 nm using a multi-mode reader (BioTek, VT, USA).

## Cell Apoptosis Assay

The percentages of apoptotic cells were quantified by flow cytometry (Beckman Coulter, CA, USA) using a PI/Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China), following the manufacturer's protocol. Briefly, the different groups of melanocytes were harvested and the cells (1x10<sup>5</sup> cells/tube) were stained with Annexin V-FITC and PI in the dark. After being washed, the cells were analysed by flow cytometry.

## RNA Sample Preparation and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from individual groups of cells using Trizol reagent (Gibco) and reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara, Kyoto, Japan), according to manufacturer's protocol. The RNA concentrations were measured using a spectrophotometer reader (NanoDrop Technologies, DE, USA). The relative levels of IL-8, IL-6 and IL-1β mRNA transcripts to the internal control GAPDH were measured by RT-qPCR using the power SYBR Green PCR master mix (Takara) and specific primers in a real-time PCR machine (Roche, Basel, Switzerland). The sequences of primers were forward 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and reverse 5'-AACCTCTGCACCCAGTTTTTC-3' for IL-8; forward 5'-GCCCCAGTACCCCCAGGAG-3' and reverse 5'-TCTGCCAGTGCCTCTTTGCT-3' for IL-6; forward 5'-CCACAGACCTTCCAGGAGAATG-3' and reverse 5'-GTGCAGTTCAGTGATCGTACAGG-3' for IL-1β; and forward 5'-AAGATCCGAGAAGAATACCCTGA-3' and reverse 5'-CTACCAACTGATGGACGGAGA-3' for β-Tubulin. The data were normalized to β-Tubulin and analysed by 2<sup>-ΔΔCt</sup>.

## Tyrosinase Activity Assay

The activity of tyrosinase in individual groups of melanocytes was measured by enzymatic assay using the Tyrosinase Activity Assay Kit (Solarbio, Beijing, China), per the supplier's protocol. Briefly, melanocytes ( $1 \times 10^5$  cells/well) were cultured in 6-well plates for specific treatment, harvested and lysed in lysis buffer on ice for 5 minutes. The tyrosinase activity was measured spectrophotometrically at an absorbance of 475 nm using a multi-mode reader (BioTek) and normalized to the protein concentration of each sample.

## Statistical Analysis

The data are presented as mean  $\pm$  standard deviation (SD). The difference among groups was analysed by one- or two-way ANOVA using SPSS 25.0. Graphs were created using GraphPad Prism 9.0. A *P*-value of  $<0.05$  was considered statistically significant.

## Results

### Characterization of Primary Melanocytes and MSC-Exo

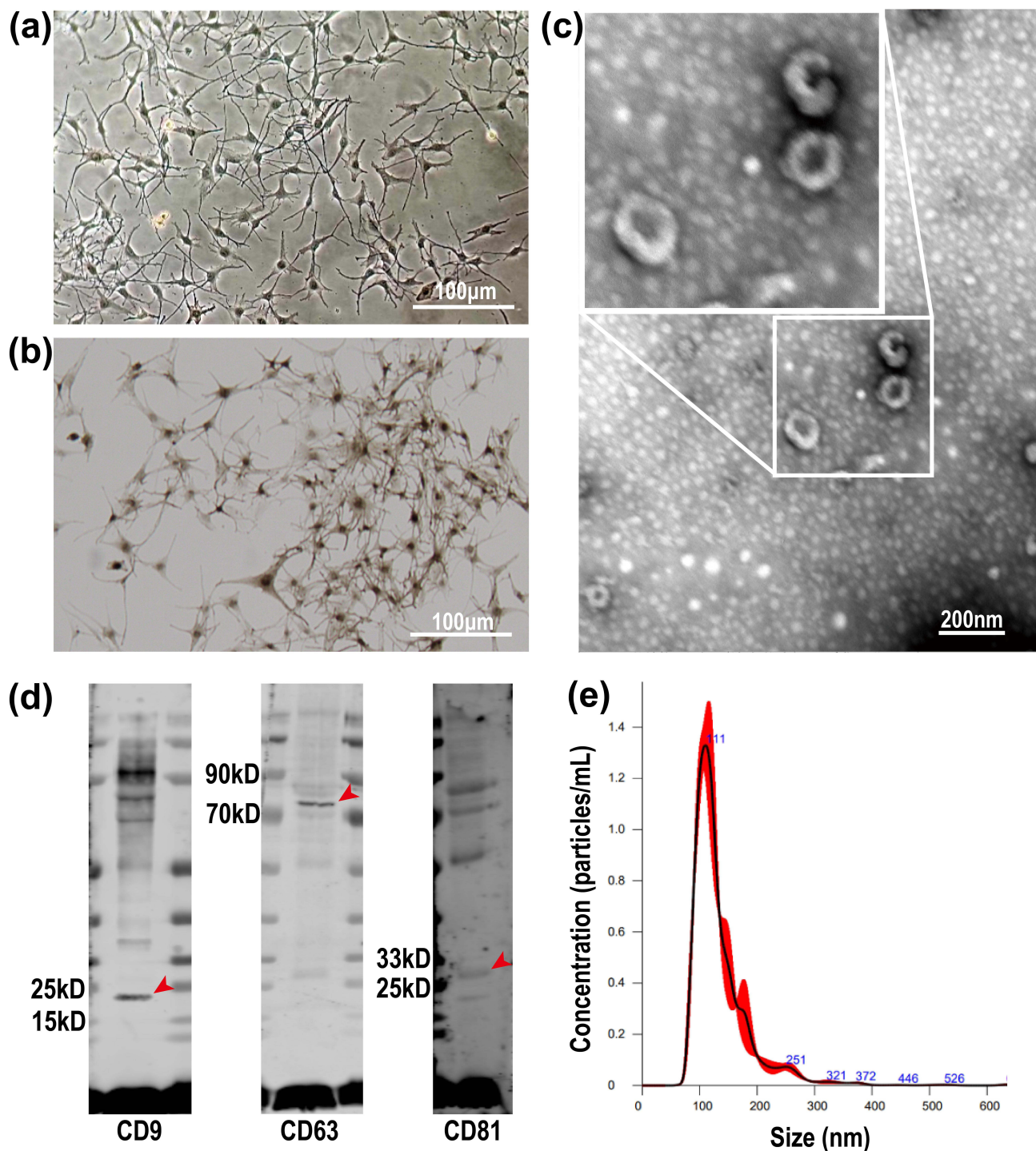
Following culture of melanocytes for 14 days, those cells displayed bipolar or multipolar morphology under a microscope (Figure 1a). L-Dopa staining revealed that the isolated melanocytes exhibited grey-brown cytosol and dendrites under a phase-contrast microscope, an indicative of positive L-Dopa staining (Figure 1b). The EVs of hUC-MSCs were isolated by sequential centrifugations and the isolated MSC-Exo had an average of about 100 nm in size (Figure 1c). TEM analysis unveiled that these MSC-Exo displayed a three-dimensional "teacup tray" shape and a double-layered membrane structure for a typical morphology (Figure 1c). Furthermore, WB revealed that these MSC-Exo contained CD9, CD63, and CD81, typical protein markers of exosome (Figure 1d). Moreover, NTA results indicated that these MSC-Exo had a peak diameter of 110 nm (Figure 1e). Hence, we successfully isolated human melanocytes and MSC-Exo.

### Pretreatment with MSC-Exo Has Opposite Effects on the H<sub>2</sub>O<sub>2</sub>-Induced Apoptosis of Human Primary Melanocytes

Next, our study tested the effect of MSC-Exo on the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of human primary melanocytes. Human primary melanocytes were pretreated with 1–40  $\mu\text{g/mL}$  MSC-Exo for 24 hours and exposed to 750  $\mu\text{mol/L}$  of H<sub>2</sub>O<sub>2</sub> for another 24 hours. The percentages of apoptotic melanocytes were determined by flow cytometry after staining them with Annexin V-FITC and PI. Quantitative analysis indicated that compared with the untreated control, pretreatment with 1  $\mu\text{g/mL}$  MSC-Exo significantly decreased the percentages of apoptotic melanocytes (Figure 2a and b). However, pretreatment with a higher dose of 5 or 10  $\mu\text{g/mL}$  MSC-Exo did not change the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of melanocytes and pretreatment with further increased doses (20, 40  $\mu\text{g/mL}$ ) of MSC-Exo significantly increased the frequency of apoptotic melanocytes in vitro.

Caspase-3 and MITF are important regulators of cell apoptosis and melanogenesis, respectively. To determine the dose effects of MSC-Exo, the levels of cleaved caspase-3 and MITF expression in the different groups of melanocytes were analysed by WB. Compared with the control, pretreatment with the indicated doses of MSC-Exo significantly reduced caspase-3 cleavage (Figure 2c). Pretreatment with a low concentration (1  $\mu\text{g/mL}$ ) of MSC-Exo had the most down-regulatory effect on caspase-3 cleavage while pretreatment with a high dose (40  $\mu\text{g/mL}$ ) of MSC-Exo achieved a little inhibitory effect on caspase-3 cleavage in the H<sub>2</sub>O<sub>2</sub>-exposed melanocytes (Figure 2c). In contrast, pretreatment with 1  $\mu\text{g/mL}$  MSC-Exo obviously increased the levels of MITF expression, but pretreatment with 40  $\mu\text{g/mL}$  MSC-Exo dramatically decreased MITF expression in the H<sub>2</sub>O<sub>2</sub>-exposed melanocytes (Figure 2c). Apparently, pretreatment with MSC-Exo had opposite effects on the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of primary melanocytes, dependent on its doses and pre-treatment with low concentration of MSC-Exo protected strongly against the H<sub>2</sub>O<sub>2</sub>-induced apoptosis and promoted melanogenesis of melanocytes. Therefore, we chose 1  $\mu\text{g/mL}$  as the intervention concentration for subsequent experiments.

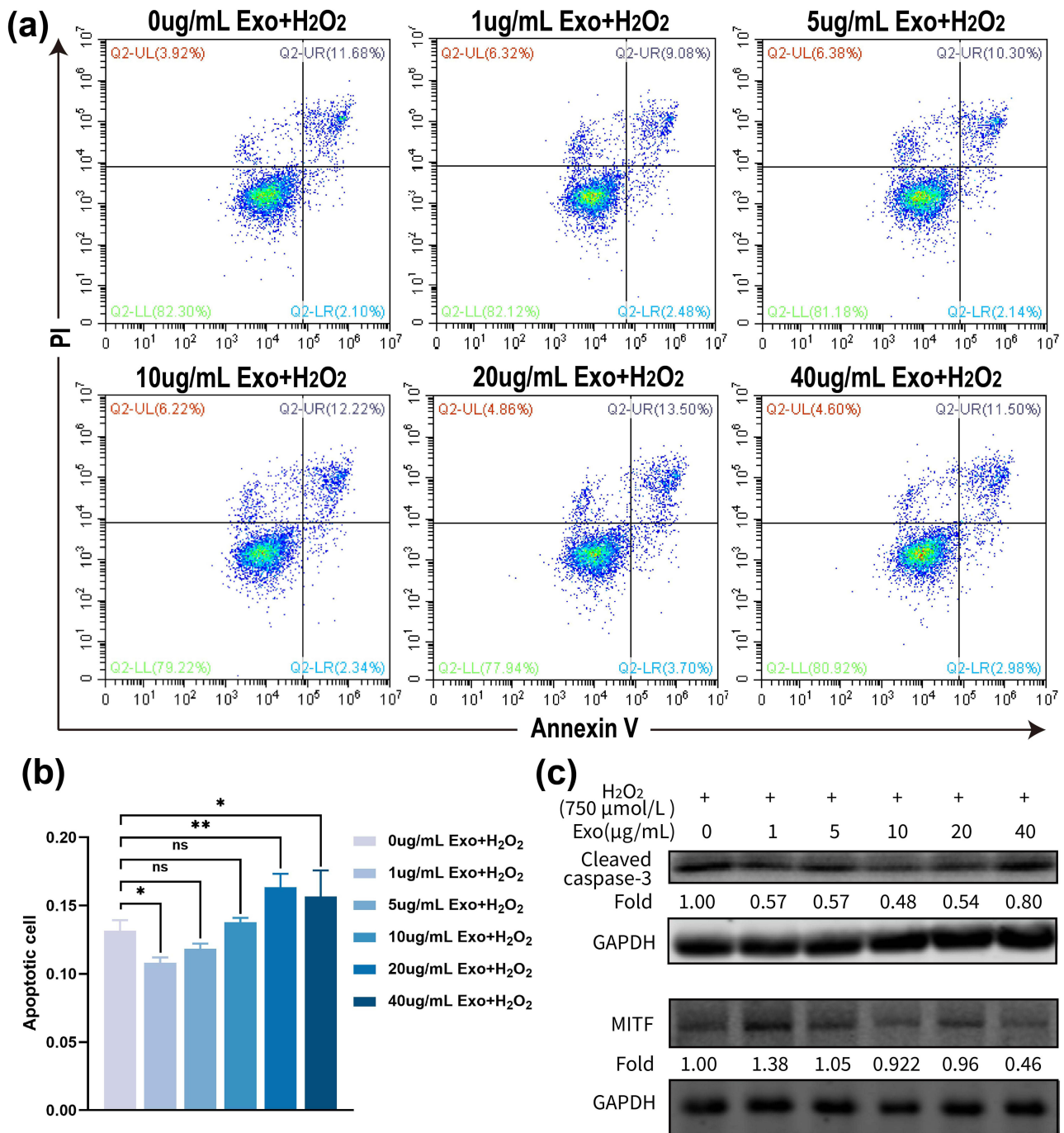




**Figure 1** Identification of primary melanocytes and characterization of exosomes from human MSCs. (a) The morphology of cultured human primary melanocytes (scale bar = 100  $\mu$ m). (b) L-Dopa staining of melanocytes (scale bar = 100  $\mu$ m). (c) TEM analysis of MSC-Exo (magnification  $\times$  80,000, scale bar = 200 nm). (d) Western blot analysis of the relative levels of CD9, CD63, and CD81 expression in MSC-Exo, the red arrow indicates the location of the target bands of three exocrine marker proteins, including CD9, CD63, and CD81. (e) NTA analysis of MSC-Exo sizes.

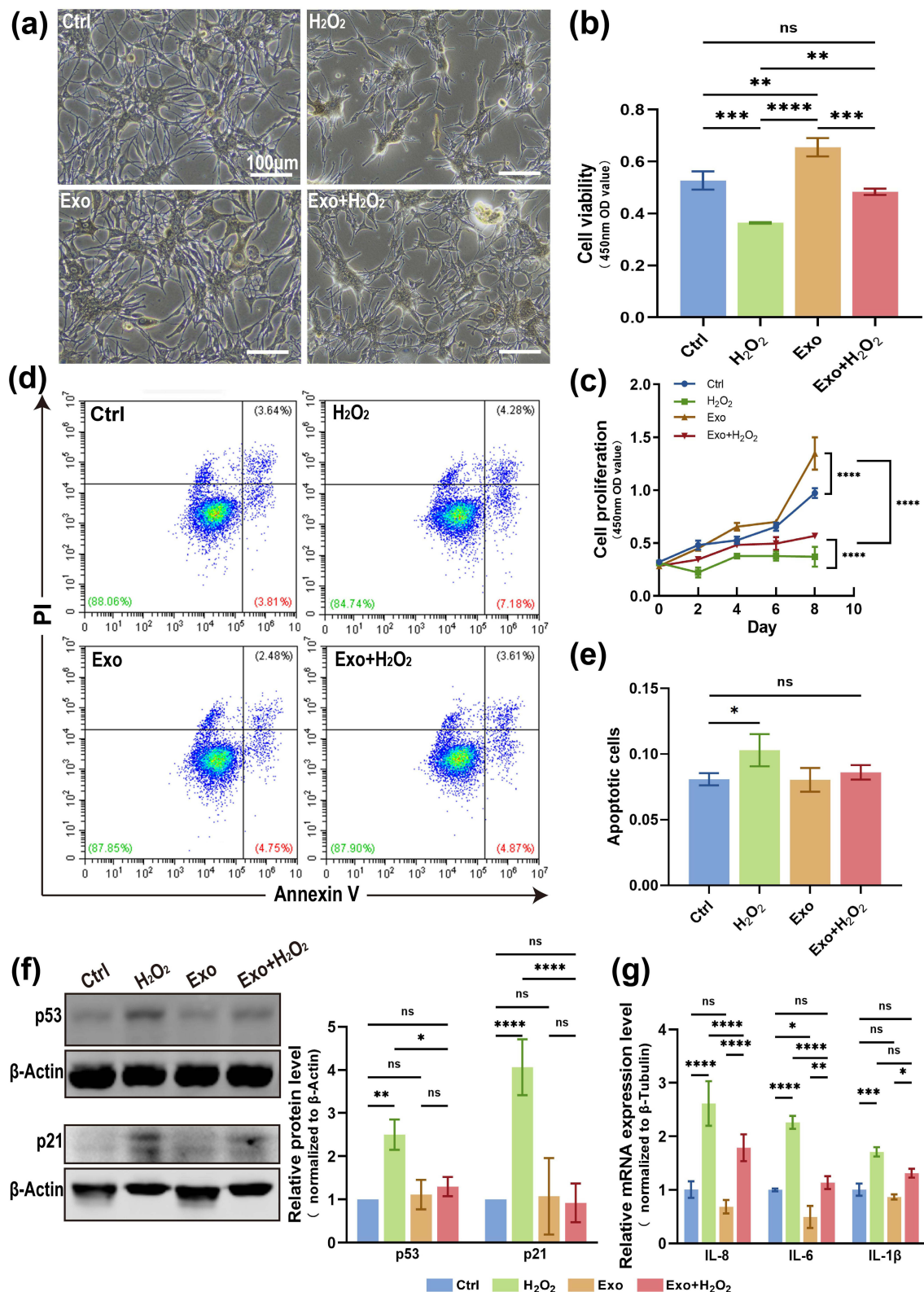
## MSC-Exo Attenuates the H<sub>2</sub>O<sub>2</sub>-Induced Cytotoxicity Against Melanocytes by Promoting Their Survival

To understand the role of MSC-Exo in regulating the survival of melanocytes, human primary melanocytes were pretreated with vehicle alone or 1  $\mu$ g/mL MSC-Exo for 24 hours and treated with vehicle alone or 750  $\mu$ mol/L of H<sub>2</sub>O<sub>2</sub> for 24 hours. As



**Figure 2** MSC-Exo have dual effects on the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against melanocytes, dependent on their doses. Human primary melanocytes were pretreated with the indicated doses of MSC-Exo for 24 hours and exposed to 750 μmol/L of H<sub>2</sub>O<sub>2</sub> for another day. The percentages of apoptotic melanocytes were analysed by flow cytometry after staining them with Annexin V-FITC and PI. (a and b) Flow cytometry and quantitative analysis of apoptotic melanocytes in the different groups. (c) Western blot analysis of cleaved caspase-3 and MITF expression. The relative quantification of the bands is shown using GAPDH as an internal reference. \*P<0.05, \*\*P<0.01. **Abbreviation:** ns, not significant.

a result, there were four groups of cells including the control (Ctrl), H<sub>2</sub>O<sub>2</sub>, Exo, Exo + H<sub>2</sub>O<sub>2</sub> groups. Morphologically, melanocytes in the control and Exo groups displayed regular cytosolic and dendritic morphology with most cells with 3–6 dendrites and some cell fusion together (Figure 3a). In contrast, the H<sub>2</sub>O<sub>2</sub> group exhibited a reduced number of cells, particularly with few cell fusion and thinner and shorter dendrites, and many cells only had 2–3 dendrites with membrane exfoliation and cell shrinkage, indicating that H<sub>2</sub>O<sub>2</sub> exposure impaired melanocytes. Pretreatment with 1 μg/mL MSC-Exo



**Figure 3** Pretreatment with MSC-Exo enhances the proliferation of melanocytes and mitigates the H<sub>2</sub>O<sub>2</sub>-induced senescence and cytotoxicity against melanocytes. Human primary melanocytes were pretreated with vehicle or 1 μg/mL MSC-Exo for 24 hours and treated with, or without, 750 μmol/L of H<sub>2</sub>O<sub>2</sub> for another 24 hours. (a) The morphology of melanocytes (scale bar = 100 μm). (b) and (c) CCK-8 analysis of the dynamic growth of melanocytes of different groups. (d) and (e) Pretreatment with MSC-Exo abrogated the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of melanocytes. (f) Western blot analysis of the relative levels of p21 and p53 expression in melanocytes. (g) RT-qPCR analysis of the relative levels of IL-8, IL-6, and IL-1β mRNA transcripts in the different groups of melanocytes. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, and \*\*\*\*P<0.0001.



obviously mitigated the damage of melanocytes and improved their morphology in the Exo+H<sub>2</sub>O<sub>2</sub> group, reflecting that pretreatment with MSC-Exo protected against the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against human primary melanocytes *in vitro*. Similarly, compared with the control, H<sub>2</sub>O<sub>2</sub> exposure significantly decreased the viability of melanocytes while pretreatment with 1 µg/mL MSC-Exo significantly increased the viability of melanocytes and abrogated the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against melanocytes (Figure 3b). A similar pattern of the dynamic melanocyte proliferation was observed by CCK-8 assays (Figure 3c). Furthermore, H<sub>2</sub>O<sub>2</sub> exposure significantly increased the frequency of apoptotic melanocytes while pretreatment with 1 µg/mL MSC-Exo promoted the survival of melanocytes regardless of H<sub>2</sub>O<sub>2</sub> exposure (Figure 3d and e). Moreover, while H<sub>2</sub>O<sub>2</sub> exposure significantly increased the relative levels of p53 and p21 protein expression, pretreatment with 1 µg/mL MSC-Exo completely abrogated the H<sub>2</sub>O<sub>2</sub>-enhanced p53 and p21 expression in melanocytes (Figure 3f). Given that inflammatory cytokines, such as, IL-8, IL-6, and IL-1β, contribute to the process of melanocyte senescence, we tested the relative levels of IL-8, IL-6, and IL-1β mRNA transcripts in the different groups of melanocytes by RT-qPCR. The results indicated that H<sub>2</sub>O<sub>2</sub> exposure significantly increased the relative levels of IL-8, IL-6, and IL-1β mRNA transcripts, relative to that in the control while pretreatment with 1 µg/mL MSC-Exo significantly decreased IL-6 mRNA transcripts and dramatically mitigated or abrogated the H<sub>2</sub>O<sub>2</sub>-enhanced IL-8, IL-6, and IL-1β mRNA transcripts in melanocytes (Figure 3g). Collectively, these data evidenced that pretreatment with MSC-Exo prevented the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and senescence of melanocytes by promoting their survival *in vitro*.

## MSC-Exo Rescues the H<sub>2</sub>O<sub>2</sub>-Inhibited Melanogenesis in Primary Melanocytes

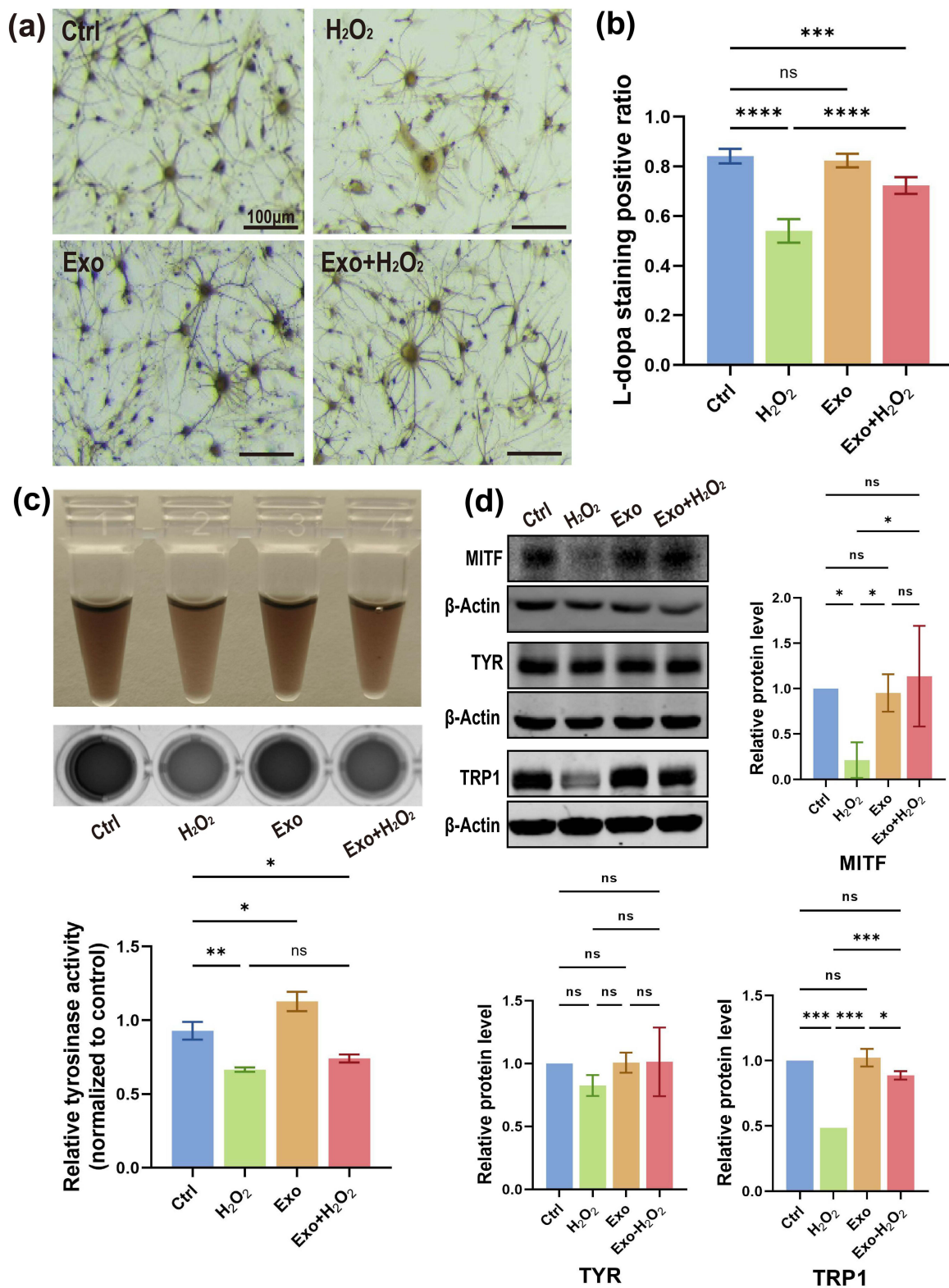
Finally, we tested the impact of pretreatment with MSC-Exo on melanogenesis of human primary melanocytes following H<sub>2</sub>O<sub>2</sub> treatment. Following culture of the different groups of cells for 48 hours, the cells were subjected to L-Dopa staining, which can quantify the levels of melanin synthesis in melanocytes.<sup>40</sup> The results displayed that the cells in the H<sub>2</sub>O<sub>2</sub> group exhibited noticeably lighter cytosolic L-Dopa staining and fewer dendrites compared to the cells in the control group while pretreatment with MSC-Exo did not significantly increase the levels of L-Dopa staining in the Exo group, but partially rescued the H<sub>2</sub>O<sub>2</sub>-reduced L-Dopa staining in the Exo+H<sub>2</sub>O<sub>2</sub> group of melanocytes (Figure 4a and b). A similar pattern of Tyrosinase activities was detected in the different groups of cells (Figure 4c). Furthermore, compared with the Control, H<sub>2</sub>O<sub>2</sub> exposure significantly reduced the relative levels of MITF, TRP1, but not TYR expression while pretreatment with MSC-Exo significantly mitigated or abrogated the H<sub>2</sub>O<sub>2</sub>-reduced MITF and TRP1 expression in melanocytes in our experimental conditions (Figure 4d). Together, these data indicated that pretreatment with MSC-Exo promoted the melanogenesis of human primary melanocytes following H<sub>2</sub>O<sub>2</sub> treatment.

## Discussion

In this study, we investigated the effects of MSC-Exo on the H<sub>2</sub>O<sub>2</sub>-affected survival of human primary melanocytes and their proliferation, apoptosis, senescence, and melanogenesis *in vitro*. The data indicated that treatment with a low dose of MSC-Exo promoted the proliferation and melanogenesis of human primary melanocytes by ameliorating the H<sub>2</sub>O<sub>2</sub>-induced apoptosis and senescence of melanocytes. Our findings suggest that MSC-Exo may be a promising therapeutic agent for vitiligo.

Vitiligo clinically displays depigmented or hypopigmented patches due to progressive destruction of melanocytes. The aetiology of vitiligo is attributed to a high genetic vulnerability, oxidative stress, inflammation, and dysregulation of senescence or apoptosis.<sup>2,41</sup> Early and aggressive treatment may prevent damage to melanocytes and/or improve their resistance to damage.

Exosome is one type of EVs and has a size of 40–160 nm in diameter. Exosomes can act as mediators of intercellular communication by carrying and exchanging bioactive components, such as proteins, cytokines and nucleic acids.<sup>22,42</sup> A recent study indicates that MSC-Exo is now widely accepted as the next generation cell-free therapy for refractory diseases.<sup>30</sup> In the field of dermatology, MSC-Exo has also been proven to have enormous potential for biomarkers and therapeutics.<sup>43</sup> Treatment with MSC-Exo has protective and promotive effects on human keratinocytes<sup>44</sup> and dermal fibroblasts<sup>45</sup> *in vitro*. Actually, co-culture of MSC with melanocytes promotes the proliferation of melanocytes.<sup>46</sup> In this study, we found for the first time, that treatment with low dose of MSC-Exo enhanced the proliferation of human primary melanocytes, besides mitigating the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against melanocytes *in vitro*. These novel findings extended previous observation and support the notion that MSC-Exo protects melanocytes from the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity.



**Figure 4** Pretreatment with MSC-Exo attenuates the H<sub>2</sub>O<sub>2</sub>-reduced melanogenesis in primary melanocytes. (a) L-Dopa staining of melanocytes (scale bar = 100 μm). (b) The percentages of positive L-Dopa staining melanocytes in different groups. (c) Tyrosinase activity of melanocytes. (d) Western blot analysis of the relative levels of MITF, TYR and TRP1 expression in the different groups of melanocytes. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, and \*\*\*\*P<0.0001.



Accumulated data support the hypothesis that apoptosis, but not necrosis, is responsible for the loss of melanocytes during the early process of vitiligo.<sup>47-49</sup> Actually, high levels of CD95L (FasL) expression are detected in the epidermis and dermis of both perilesional and non-lesional skin of active vitiligo patients, suggesting that the FasL/Fas-related apoptosis may be crucial for melanocyte destruction and the pathogenesis of active vitiligo.<sup>50</sup> Compared to healthy controls, perilesional melanocytes from unstable vitiligo display higher levels of caspase-3 expression following apoptosis is stimuli.<sup>51</sup> It is notable that MSC-Exo have potent anti-apoptotic effect *in vitro*<sup>52,53</sup> and *in vivo*.<sup>54,55</sup> In this study, we found that pretreatment of melanocytes with low dose of MSC-Exo had reversal effects on the H<sub>2</sub>O<sub>2</sub>-induced caspase-3 cleavage and melanocyte apoptosis. As previous studies found that MSC-Exo could repair oxidative stress-induced damage in keratinocytes and skin of animal model through adaptive regulation of NRF2 defense system and NRF2/HO-1 signaling pathway is involved in the mitigation of H<sub>2</sub>O<sub>2</sub>-mediated apoptosis in human keratinocytes,<sup>32,56</sup> we suggest that MSC-Exo may mitigate the H<sub>2</sub>O<sub>2</sub>-triggered apoptosis of melanocytes through the NRF2/HO-1 pathway.

However, our flow cytometry results indicated that higher doses (20 and 40 µg/mL) of MSC-Exo had a pro-apoptotic effect on cells with H<sub>2</sub>O<sub>2</sub> exposure, which was in accordance with a previous study in cardiomyocytes.<sup>52</sup> Combined with our WB results that lower doses of exosomes inhibited the production of apoptosis-associated cleaved caspase-3 induced by H<sub>2</sub>O<sub>2</sub>, we hypothesized that the pro-apoptotic effect of high concentrations of MSC-Exo might be mediated through the caspase-3-independent pathway in melanocytes, such as excessive MSC-Exo intake by melanocytes leading to overloading of cells and thus activating other apoptotic pathways. More experiments are needed to prove this hypothesis.

Previous studies have shown that melanocyte senescence participates in the early pathogenic process of vitiligo and the persistent aggravation of damage will eventually lead to death and destruction of melanocytes.<sup>13,37</sup> In this study, we found that pretreatment with MSC-Exo dramatically abrogated or mitigated the H<sub>2</sub>O<sub>2</sub>-enhanced P53 and p21 expression, IL-8, IL-6 and IL-1β mRNA transcription in melanocytes. The upregulated p53 expression is previously observed in melanocytes of non-lesional vitiligo skin,<sup>14,57</sup> and p53 can induce downstream p21 expression. Furthermore, during the process of senescence, stimuli can also induce the overexpression of the senescence-associated secretory phenotype (SASP)-related cytokines including IL-6 and IL-8.<sup>58</sup> Given that up-regulated p53 and downstream p21 expression are hallmarks of cell senescence, the significantly attenuated p53 and p21 expression by MSC-Exo, together with the decreased levels of IL-8, IL-6 and IL-1β expression, clearly indicated that pretreatment with MSC-Exo inhibited and prevented the H<sub>2</sub>O<sub>2</sub>-induced senescence of melanocytes. We speculate that MSC-Exo may inhibit the H<sub>2</sub>O<sub>2</sub>-induced p53-p21 expression and cell senescence in human melanocytes. We are interested in further investigating the hypothesis in the future studies.

Melanogenesis is a complicated process and regulated by multiple genes and signalling pathways.<sup>59</sup> The MITF is a key transcription factor regulating the survival and melanogenesis of melanocytes and the expression of downstream proteins including TYR, TRP-1, and TRP-2.<sup>60</sup> A previous study has shown that exosomes, such as keratinocyte derived-exosome (KC-Exo), can regulate melanogenesis and miRNA in KC-Exo regulates pigmentation in melanocytes, contributing to the pathogenesis of pigmentation-related diseases.<sup>61</sup> KC-Exo containing miRNAs and other soluble factors can mediate the communication between keratinocytes and melanocytes in pigmentation modulation by regulating the expression of MITF, TYR, TRP1 and TRP2.<sup>62,63</sup> In this study, we found that pretreatment with MSC-Exo significantly rescued the H<sub>2</sub>O<sub>2</sub>-decreased MITF and TRP1 expression as well as tyrosinase activities in melanocytes. Therefore, in the early stage of melanocyte injury process induced by oxidative stress in patients with vitiligo, external or systematic use of MSC-Exo may promote the functional recovery of melanocyte melanogenesis in patients with vitiligo.

These novel data indicated that MSC-Exo enhanced the melanogenesis of melanocytes following H<sub>2</sub>O<sub>2</sub> treatment. Hence, therapeutic strategies to enhance MITF and other melanogenesis-related protein expression as well as their activities may be valuable for the intervention of vitiligo.<sup>64</sup>

Exosomes have already become promising multipotent frontiers in dermatology and cutaneous medical aesthetics.<sup>65</sup> Cryogel wound dressing OxOBand loaded with exosomes from adipose-derived stem cells has been demonstrated to facilitate wound healing in rat model of diabetic wound ulcer.<sup>66</sup> Microneedle device integrated with MSC-Exo has been shown to promote pigmentation and hair regrowth in a mouse model.<sup>67</sup> The available findings suggest that exosomes, especially MSC-Exo, are highly bioactive and may be used as a potential therapeutic option for the treatment of vitiligo.

## Conclusion

In summary, the results from this study indicated that pretreatment with MSC-Exo ameliorated the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity against human primary melanocytes by promoting the proliferation and melanogenesis of human primary melanocytes and attenuating the H<sub>2</sub>O<sub>2</sub>-induced apoptosis and senescence of melanocytes. The treatment of vitiligo has always been challenging. It is well known that the key to the treatment of vitiligo is the restoration of melanocyte activity and melanin synthesis. These findings indicate that MSC-Exo may be a potential novel cell-free therapeutic strategy for the intervention of vitiligo.

We recognized that our study had limitations. The current study only preliminarily explored the effect of MSC-Exo on melanocytes. Further elucidation of the therapeutic efficacy of MSC-Exo and the specific mechanisms underlying the action of MSC-Exo in regulating the survival and melanogenesis of melanocytes, particularly for those from vitiligo patients or in animal models of vitiligo are warranted.

## Ethics Statement

The study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. The patients provided their written informed consent before participating in this study. Our study was complied with the Declaration of Helsinki.

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## Disclosure

The authors declare no conflicts of interest in this work.

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