A safety consideration of mesenchymal stem cell therapy on COVID-19

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ABSTRACT

Due to the multi-potential differentiation and immunomodulatory function, mesenchymal stem cells (MSCs) have been widely used in the therapy of chronic and autoimmune diseases. Recently, the novel coronavirus disease 2019 (COVID-19) has grown to be a global public health emergency but no effective drug is available to date. Several studies investigated MSCs therapy for COVID-19 patients. However, it remains unclear whether MSCs could be the host cells of SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) and whether they might affect the SARS-CoV-2 entry into other cells. Here, we report that human MSCs barely express ACE2 and TMPRSS2, two receptors required for the virus endocytosis, indicating that MSCs are free from SARS-CoV-2 infection. Furthermore, we observed that MSCs were unable to induce the expression of ACE2 and TMPRSS2 in epithelial cells and macrophages. Importantly, under different inflammatory challenge conditions, implanted human MSCs failed to up-regulate the expression of ACE2 and TMPRSS2 in the lung tissues of mice. Intriguingly, we showed that a SARS-CoV-2 pseudovirus failed to infect MSCs and co-cultured MSCs did not increase the risk of SARS-CoV-2 pseudovirus infection in epithelial cells. All these results suggest that human MSCs have no risk of assisting SARS-CoV-2 infection and the use of MSCs as the therapy for COVID-19 patients is feasible and safe.

1. Introduction

SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), a recently identified coronavirus, causes severe pneumonia named COVID-19 (Coronavirus Disease In 2019). SARS-CoV-2 shares 85% identity with human SARS-CoV (Chan et al., 2020) and spreads in person-to-person via droplets from coughing; sneezing or direct contact. Angiotensin-converting enzyme 2 (ACE2) is the main host cell receptor for the SARS-CoV-2 entry (Xu et al., 2020; Zhou et al., 2020; Wrapp et al., 2020; Lan et al., 2020). Accumulating evidence suggests that the expression of ACE2 protein is mainly at the epithelia of the lung, small intestine and respiratory tract, which provide possible entry routes for SARS-CoV-2 (Xu et al., 2020; Hamming et al., 2004). Moreover, Hoffmann et al revealed that the cellular transmembrane serine protease 2

(TMPRSS2) is also essential for SARS-CoV-2 spike protein priming to enter the host cells (Hoffmann et al., 2020). Therefore, both ACE2 and TMPRSS2 have been regarded as receptors for the virus and their expression in human tissues predicts the potential infection ability in COVID-19 patients.

Currently, the cure of COVID-19 is essentially dependent on the patient's immune system and no specific drugs are available. Several drugs, including chloroquine, remdesivir, and lopinavir, failed to treat COVID-19 patients. Vaccines against SARS-CoV-2 are tightly under development and are expected to execute clinical trials (Zhu et al., 2020; Wang et al., 2020). Nevertheless, it is in urgent demand for the therapy of COVID-19 in the current situation. Mesenchymal stem cells (MSCs) are widely distributed with a potential ability for multiple differentiation and immune regulation in the body (Galipeau and Sensébé, 2018).

MSCs have been used in therapy for several diseases (Connick et al., 2012; Wilson et al., 2015; Li et al., 2017). Numerous studies have reported that MSCs function in a pluripotent way including reducing inflammation, modulating immune responses, promoting tissue regeneration and inhibiting tumor progression (D'Souza et al., 2015; Zaher et al., 2014; Tan et al., 2014). Recently, several groups confirmed that MSCs had clinical remission for COVID-19 patients without observed adverse effects (Zhao et al., 2020; Liang et al., 2020; ClinicalTrials.gov, 2020a; Meng, 2020; Atluri, 2020). To our knowledge, three clinical trials are underway to explore MSC-based treatment for COVID-19 infection (ClinicalTrials.gov, 2020b, 2020c, 2020d). A question was raised about MSC therapy for either the possibility of SARS-CoV-2 infection on MSCs per ser or the inducible infection of other cells. In this context, we sought to examine the constitutive or inducible expression of ACE2 or TMPRSS2 during the MSC therapy process.

Herein we report that MSCs from different organs or donors are negative for ACE2 and TMPRSS2, suggesting that MSCs are free from COVID-19 infection. By co-culturing with epithelial cells and macrophages, we found that MSCs were unable to induce ACE2 and TMPRSS2 expression in these cells. Furthermore, we observed that injecting human MSCs affect no expression of ACE2 and TMPRSS2 in the lung of LPS-challenged mice. Both cellular and animal results suggest that MSC therapy has no side effect on the risk of SARS-CoV-2 infection.

2. Materials and methods

2.1. Cell culture

Mesenchymal stromal cells were isolated with high efficiency from humans under the agreement of informed consent in the hospital and expanded in vitro with Eagle's minimal essential medium, supplemented with 10% fetal bovine serum and $1 \times$ L-glutamine. The Institution Review Board of Tsinghua University approved the entirety of the project and found it concurrent with all related protocols.

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected from a healthy man by using a Ficoll-Hypaque gradient centrifugation procedure (GE Healthcare, Sweden) (Sashchenko et al., 1993). PBMCs were cultured in RPMI-1640 supplemented with 10% FBS, IL-2 (50 ng/ml) and 1% penicillin/streptomycin.

Macrophages were isolated from bone marrow collected from C57B1/6 male mice. The detailed process follows a protocol in the literature (Manzanero, 2012). Macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, macrophage colony-stimulating factor (M-CSF, 50 ng/ml) and 1% penicillin/streptomycin.

293 T and Beas-2B cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. NCM460 cell was maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were cultured at 37 °C with 5% CO_2. Media and serum were purchased from Thermo Fisher (Gibco, USA).
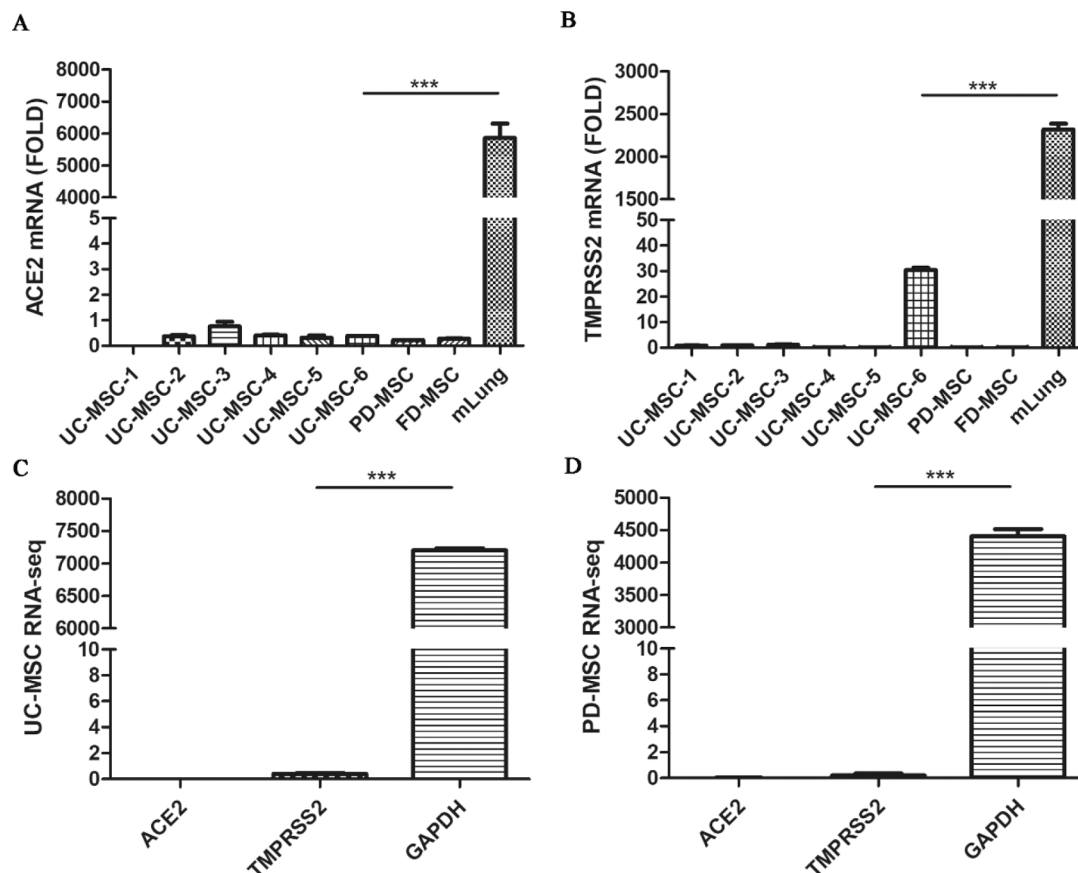
Fig. 1. Negative expression of ACE2 and TMPRSS2 in MSCs. A. the mRNA level of ACE2 in MSCs from different sources, including umbilical cords from six donors, placenta from one donor and adipose from one donor. B. the mRNA level of TMPRSS2 in MSCs from the same sources with A. C-D. RNA-seq analysis of MSCs isolated from the human umbilical and placenta of the same donor. UC-MSC represented umbilical cords-derived MSC. PD-MSC represented placenta-derived MSC. FD-MSC represented adipose-derived MSC. ***, p < 0.001. β-Actin was used as an internal control to normalize the fold changes in A and B. The mRNA of mouse lung (mLung) was used as a positive control. GAPDH was used as a reference for the RNA-seq analysis in C and D.
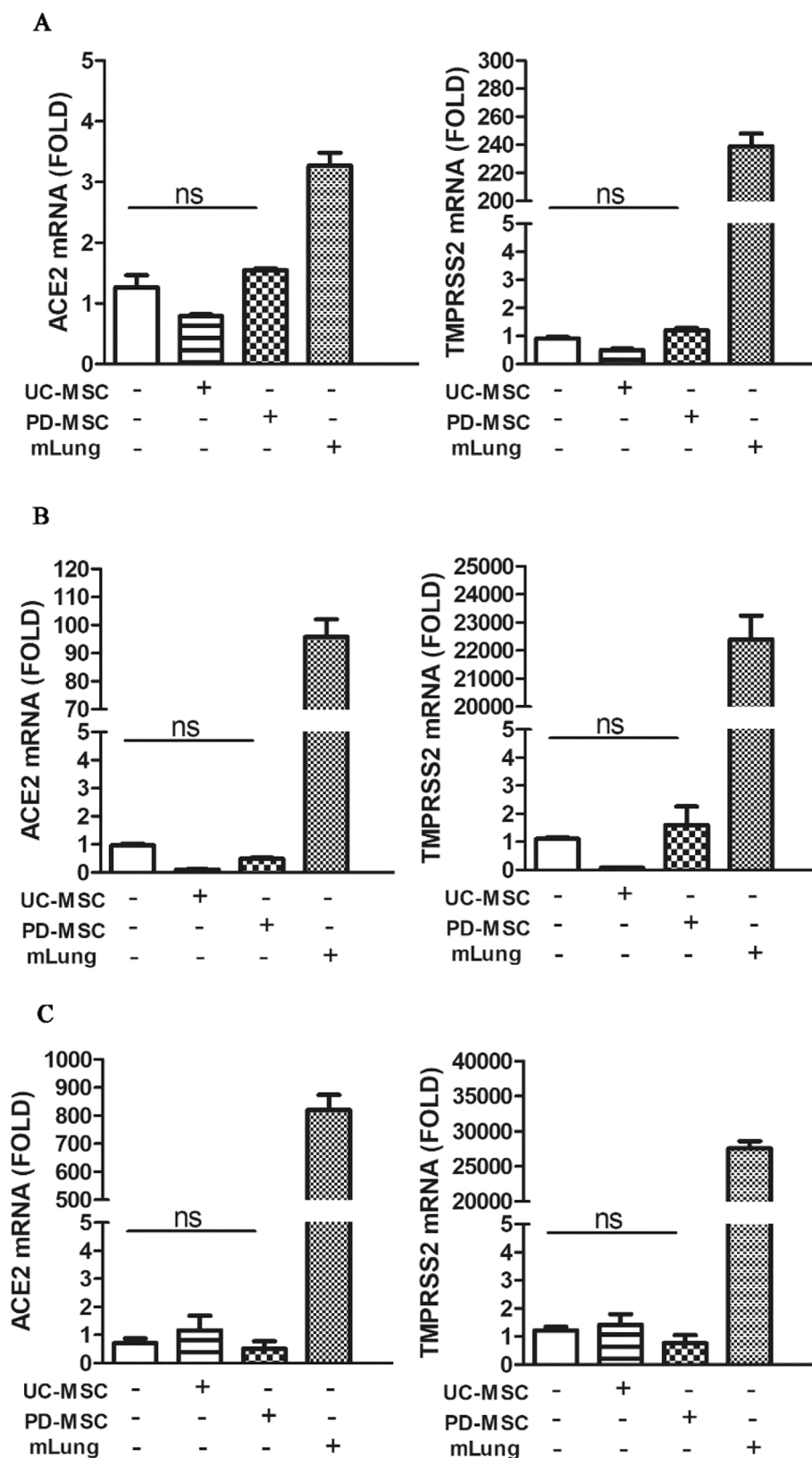
Fig. 2. MSCs induce neither ACE2 nor TMPRSS2 expression in epithelial cells and macrophages. Co-cultured UC-MSC and PD-MSC failed to influence the expression of ACE2 and TMPRSS2 in human kidney epithelial 293 T cell line (A), human colon mucosa NCM460 cell line (B), human lung epithelial Beas-2B cell line (C), human PBMC (D) and mouse macrophages (E). The mRNA of mouse lung (mLung) was used as a positive control. ns, not significantly.
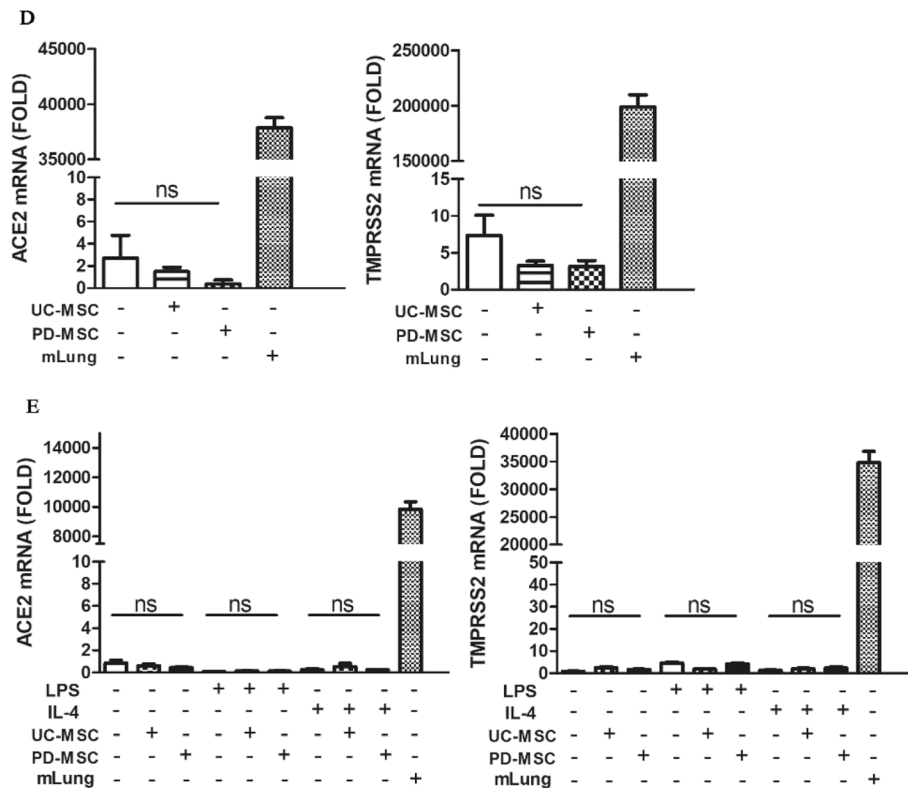
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Fig. 2. (*continued*).

2.2. Co-culture assay

Different cell lines including 293 T, NCM460, Beas-2B and PBMC cells (lower chamber) were co-cultured with MSCs (upper chamber) at the density of 2×10^5 using Transwell BD Matrigel (Costar, 3450). After 3 days, cells were harvested for RNA isolation.

For macrophages co-cultured with MSCs, we inoculated 2×10^5 MSCs at the upper chamber and 1×10^6 M0 macrophage at the lower chamber. In one experiment, macrophages were harvested for RNA isolation after three day's co-culture. In another experiment, MSCs were removed and macrophages were induced M1 or M2 macrophages by adding LPS (100 ng/ml) or IL-4 (20 ng/ml) for 24 h.

2.3. Animal assay

For acute lung injury (ALI) model, 5 mg/kg LPS were intraperitoneally injected into 8-week-old C57B1/6 male mice. Each experimental group consisted of five mice. Mice were pretreated with 1×10^5 MSCs by tail vein injection or 1×10^6 MSCs by subcutaneous injection for 18 h and were object to LPS challenge for 6 h. Then mice were re-treated with the same doses of MSCs. All mice were sacrificed on the third day after LPS challenge. The lungs from each mouse were collected for RNA extracting.

For acute lung injury model, 10 Gy X-ray irradiation was applied for mice. Then treated mice were cured with 2×10^4 UC-MSCs by tail vein injection. Each experimental group consisted of three mice. After 6 days, mice were sacrificed and lung tissues were collected.

For the pulmonary fibrosis model, 2 mg/kg bleomycin (BLM) was intratracheally instilled into C57B1/6 male mice. The treated mice were subject to therapy with 300ul MSCs-supernatant three times by tail vein injection on the 7th day, 15th day and18th day after BLM challenge. Each experimental group consisted of five mice. All mice were sacrificed on the 24th day after BLM injection. All the experimental procedures were approved by the Animal Ethics Committee of Tsinghua University.

2.4. Real-time reverse transcription (RT)-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). Reverse transcription was done using a Quant script RT kit (TIANGEN Biotech, China). Real-time PCR was performed using a Talent qPCR PreMix (SYBR Green) kit (TIANGEN Biotech, China) using the following conditions: denature, 95 °C, 5 s; annealing and extension, 60 °C, 15 s. Primers used for the human/mouse ACE2 gene were 5′- GAGATGG-CAAGAGCAAA-3′ and 5′-CCTCACATAGGCATGAAGATG-3′. Primers used for the human/mouse TMPRSS2 gene were 5′-CGCCA-GAGCAGGATTGTGGG-3′ and 5′- CCCGCAAATGCCGTCCA-3′. Primers used for the human ACTIN gene were 5′- GTGTGTGACAATGGCTCTGG-3′and 5′- TCCCAGTTGGTGATGATGCC-3′. Primers used for the mouse ACTIN gene were 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT3′.

2.5. RNA-seq analysis

Human MSCs of umbilical cord (UC-MSC-5) and MSCs of placenta (PD-MSC) from the same donor were prepared for RNA-seq analysis. Samples were sent to the company and subject to 10 × RNA-seq analysis. A total of 6500 MSCs were captured and 56,650 raw reads were obtained.

2.6. Pseudovirus infection assay

Human MSCs were incubated with pseudovirus at a density of 1×10^4 using 96 well cell culture cluster (Costar, 3599). After 48 h, cells were harvested for luciferase experiments (Vigorous, Dual-Lucy Assay Kit).

Different cell lines including 293 T, NCM460 and Beas-2B cells (lower chamber) were co-cultured with MSCs (upper chamber) at the density of 2×10^5 using Transwell BD Matrigel (Costar, 3450). After 3 days, cells were incubated with pseudovirus at the density of 1×10^4 using 96 well cell culture cluster (Costar, 3599). After 48 h, cells were
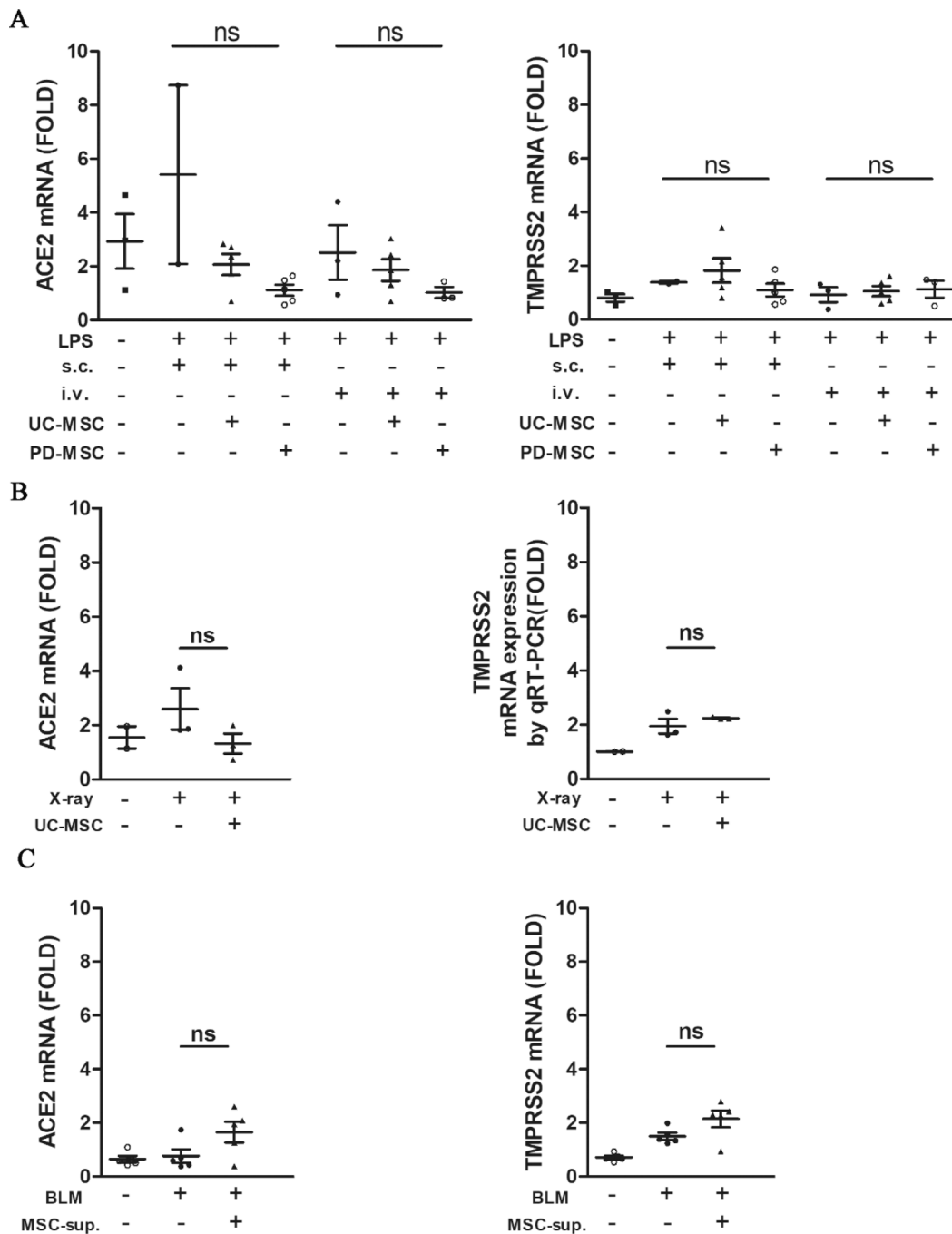
Fig. 3. ACE2 and TMPRSS2 are not influenced by MSCs in the lung of acute inflammation-challenged mice. A. the mRNA levels of ACE2 and TMPRSS2 in the lung of LPS-challenged mice after MSCs therapy by subcutaneous injection (s.c.) or tail intravenous injection (i.v.). B. the mRNA levels of ACE2 and TMPRSS2 in the lung of X-ray challenged mice after UC-MSCs therapy by tail intravenous injection. C. the mRNA levels of ACE2 and TMPRSS2 in the lung of BLM challenged mice after MSC supernatant (MSC-sup.) therapy. ns, not significantly.

harvested for luciferase experiments (Vigorous, Dual-Lucy Assay Kit).

2.7. Statistical analysis

Data were presented as mean $+/-$ standard deviation. Significant differences between groups were determined using a Student's t-test. ***, $p < 0.001$; *, $p < 0.05$.

3. Results

3.1. Negative expression of ACE2 and TMPRSS2 in MSCs from different organs or donors

We isolated and cultured different sources of human MSCs, including umbilical cords-derived MSCs (UC-MSCs) from six donors, placenta-derived MSC (PD-MSC) from one donor and adipose-derived MSC (FD-MSC) from one donor (Fig. 1). To address whether these MSCs have any potential for the SARS-Cov-2 infection, we examined the expression of ACE2 and TMPRSS2, two receptors of epithelial cells responsible for the
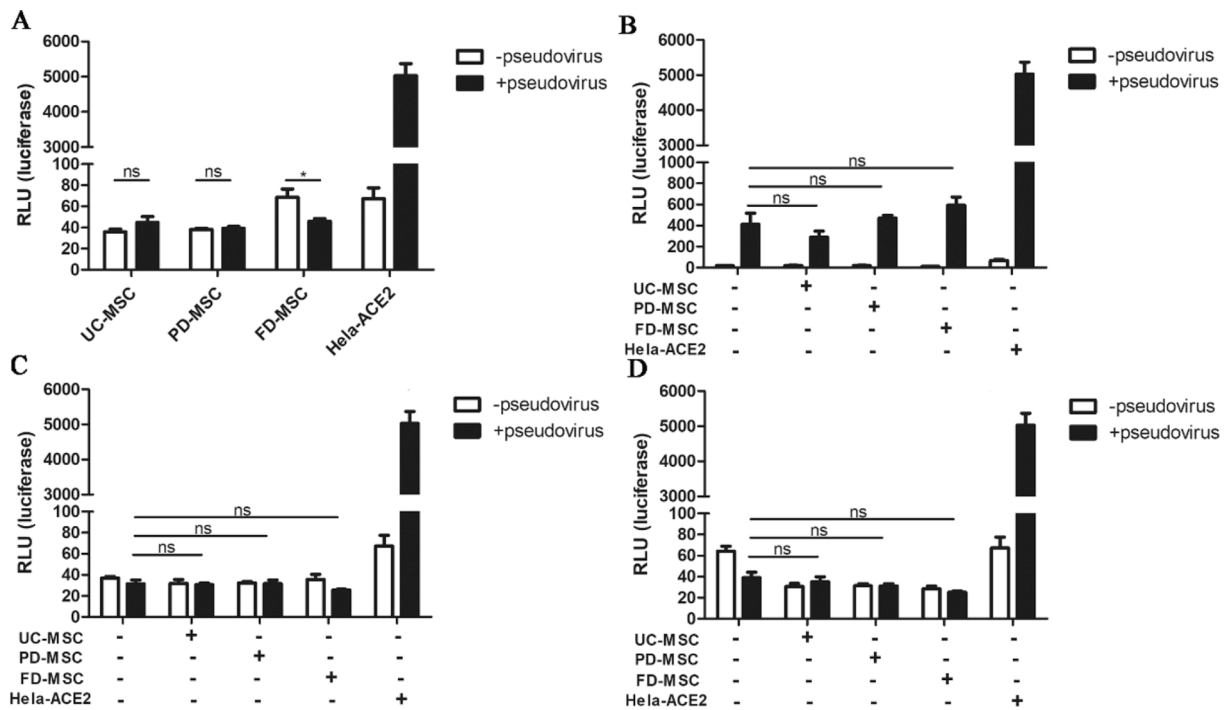
Fig. 4. The effect of a pseudovirus of SARS-CoV-2 on MSCs and MSC co-cultured cells. A. MSCs are unable to be infected by the pseudovirus of SARS-CoV-2. The luciferase activity was measured in MSCs with SARS-CoV-2 pseudovirus infection. B-D. Co-cultured MSCs have no effect on the infection of the pseudovirus in human kidney epithelial 293 T (B), human colon mucosa NCM460 (C) and human lung epithelial Beas-2B (D) cell lines. UC-MSC: umbilical cords-derived MSC, PD-MSC: placenta-derived MSC, FD-MSC: adipose-derived MSC. *, $p < 0.05$; ns, not significantly. The ACE2-overexpressed Hela cell line (Hela-ACE2) was used as a positive control.

virus entry (Hoffmann et al., 2020; Xu et al., 2020; Zhou et al., 2020; Wrapp et al., 2020; Lan et al., 2020). A real-time PCR analysis showed that all these MSCs expressed an extremely low level of ACE2 ($\Delta Ct =$ 40), compared with the mouse lung tissue (6000-fold, $p < 0.001$) (Fig. 1A). Concurrently, we observed that the mRNA level of TMPRSS2 remained at a low level except for one donor with a significantly high level (Fig. 1B). From these results, we concluded that most of MSCs from different donors expressed little ACE2 and TMPRSS2. However, it may be useful to examine their expression before planning to use these cells to exclude the rare case of elevated expression.

To validate our results, we performed an RNA-seq analysis in MSCs isolated from the human umbilical cord and placenta of the same donor. The results showed that the mRNA levels of both ACE2 and TMPRSS2 remained at an extremely low number of reads (Fig. 1C-D). Taken together, our results clearly showed that MSCs from different sources are ACE2 or TMPRSS2 negative.

3.2. MSCs induce neither ACE2 nor TMPRSS2 expression in epithelial cells and macrophages

To eliminate the concern that MSCs may influence the SARS-CoV-2 infection in other cells during MSC therapy, we co-cultured MSCs with different cells and examined the levels of ACE2 and TMPRSS2. We selected epithelial cells, macrophages and PBMC from humans or mice. Real-time PCR analyses indicated that co-cultured UC-MSC and PD-MSC did not influence the expression of ACE2 and TMPRSS2 in epithelial cells, including human kidney epithelial 293 T, human colon mucosa NCM460 and human lung epithelial Beas-2B cell lines (Fig. 2A-C).

Subsequently, we confirmed that the mRNA levels of ACE2 and TMPRSS2 were not upregulated in human peripheral blood mononuclear cells (PBMC) and mouse macrophages, which were ACE2 and TMPRSS2 negative (Fig. 2D-E). Furthermore, co-cultured UC-MSC and PD-MSC had no effect on the expression of ACE2 and TMPRSS2 in macrophages under LPS and IL-4 challenges. All these results suggested

that MSCs were unable to upregulate the expression of ACE2 and TMPRSS2 in epithelial cells and macrophages.

3.3. ACE2 and TMPRSS2 are not induced by MSCs in the lung of LPS-challenged mice

We further addressed whether UC-MSCs or PD-MSCs affect the expression of ACE2 and TMPRSS2 in a therapeutic model for LPS-induced acute inflammation in mice. We isolated the total mRNA from the lung and performed a real-time PCR analysis. The results showed that UC-MSC or PD-MSC injection had no impact on the mRNA levels of ACE2 and TMPRSS2 in the lung of LPS-challenged mice (Fig. 3A). Importantly, the injection strategies including tail vein injection and subcutaneous injection did not alter the effect of MSCs on ACE2 and TMPRSS2 expression (Fig. 3A). In other inflammation models, we observed that the expression of ACE2 and TMPRSS2 in the lung of mice challenged by X-ray and BLM remained unchanged after MSC therapy (Fig. 3B-C). These results were consistent with the observations in our aforementioned in vitro co-culture experiments. Taken together, our study provided evidence that MSC therapy influences neither ACE2 nor TMPRSS2 expression.

3.4. A SARS-CoV-2 pseudovirus failed to infect MSCs and co-cultured MSCs did not increase the risk of SARS-CoV-2 pseudovirus infection in epithelial cells

To validate the conclusion that MSCs are unable to be infected by SARS-CoV-2, we performed an infection assay by using a pseudovirus which could produce luciferase. The luciferase activity was measured and the results showed that the pseudovirus of SARS-CoV-2 failed to infect different sources of human MSCs (Fig. 4A). But Hela-ACE2, an ACE2 overexpression cell line based on Hela cells, was infected by the pseudovirus (Fig. 4A). Furthermore, the results indicated that co-cultured UC-MSC, PD-MSC and FD-MSC did not promote the luciferase

signal in the pseudovirus infected 293 T, NCM460 and Beas-2B cell lines (Fig. 4B-D). In summary, these results confirmed that MSCs are free from SARS-CoV-2 infection and MSCs have no effect on increasing the risk of SARS-CoV-2 infection in epithelial cells.

4. Discussion

Recently, the COVID-19 pandemic caused by SARS-CoV-2 is a global issue leading to the high rate of death among aged people, especially those with chronic diseases (Zhu et al., 2020). It has been reported that SARS-CoV-2 infects cells through the binding between the spike glycoprotein of coronavirus and the host cellular SARS-CoV-2 receptor, ACE2 (Xu et al., 2020; Zhou et al., 2020; Wrapp et al., 2020; Lan et al., 2020). TMPRSS2 is another necessary protein required to assist the entry of SARS-CoV-2 into cells by ACE2 cleavage, which promotes SARS-CoV-2 spike protein cleavage to activate the spike protein for membrane fusion (Hoffmann et al., 2020). Therefore, the expression levels of ACE2 and TMPRSS2 in cells are related to the infection efficiency of SARS-CoV-2 in humans.

Several up-to-date studies demonstrated the therapeutic effects of MSCs on COVID-19 (Zhao et al., 2020; Liang et al., 2020; ClinicalTrials. gov, 2020a). However, little is known about whether MSCs could be potential target cells or MSC therapy could raise the possibility of SARS-CoV-2 infection. Recently, two related studies showed that MSCs were ACE2 and TMPRSS2 negative by RNA-sequence and qPCR analyses (Zhao et al., 2020; Ma et al., 2020). However, the data is still deficient and more documents are needed.

In this study, we confirmed that most of MSCs from different organs or donors barely expressed ACE2 or TMPRSS2 (Fig. 1), implying that MSCs are unable to be infected by SARS-CoV-2. However, our results showed that TMPRSS2 was increasingly expressed in UC-MSC from one of the donors (Fig. 1B). This raised a concern that different donors may have a diversified expression pattern of TMPRSS2. Nevertheless, our results showed a ubiquitously low expression pattern of ACE2 among different donors (Fig. 1A). Thus, it is necessary to select MSCs according to the low level of ACE2 and TMPRESS2 in the therapeutic practice to ascertain the safety by excluding the rare case of SARS-CoV-2 infection.

To our knowledge, this is the first study clearly confirming that MSCs were unable to induce the expression of ACE2 and TMPRSS2 in epithelial cells and macrophages (Fig. 2). We also observed that MSCs failed to induced ACE2 and TMPRSS2 expression in the lungs from three inflammation injury mouse models (Fig. 3). Moreover, we confirmed that MSCs are free from SARS-CoV-2 infection by a pseudovirus infection assay (Fig. 4A) and co-cultured MSCs did not increase the risk of SARS-CoV-2 infection in epithelial cells (Fig. 4B-D). This study provided sufficient evidence for the safety of MSC therapy. We expect MSCs have no side effects on promoting the risk of SARS-CoV-2 infection based on their inability to inducing the expression of ACE2 and TMPRSS2, two receptors are responsible for SARS-CoV-2 infection. Although the clinical evidence is still needed, this study provided strong evidence that MSC therapy has no adverse effect on promoting SARS-CoV-2 infection. We prospected that MSCs could be used in the COVID-19 treatment without concerns about increased virus infection.

Acknowledgments

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