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Gap junction mediated cell-cell interaction between transplanted mesenchymal stem cells and vascular endothelium in stroke

[Running head of the title: Mesenchymal stem cells and vascular endothelium]

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Key words: mesenchymal stem cell; cell therapy; immunosuppression; gap junction; COVID-19.

Abstract

We have shown that transplanted bone marrow mononuclear cells (BM-MNC), which are a cell fraction rich in hematopoietic stem cells, can activate cerebral endothelial cells via gap junction mediated cell-cell interaction. In this article, we investigated such cell-cell interaction between mesenchymal stem cells (MSC) and cerebral endothelial cells. In contrast to BM-MNC, MSC we observed suppression of vascular endothelial growth factor (VEGF) uptake into endothelial cells and transfer of glucose from endothelial cells to MSC *in vitro.* The transfer of such a small molecule from MSC to vascular endothelium was subsequently confirmed *in vivo*, and was followed by suppressed activation of macrophage/microglia in stroke mice. The suppressive effect was absent by blockade of gap junction at MSC. Furthermore, gap junction mediated cell-cell interaction was observed between circulating white blood cells (WBC) and MSC. Our findings indicate that gap junction mediated cell-cell interaction is one of the major pathways for MSC-mediated suppression of inflammation in the brain following stroke and provides a novel strategy to maintain blood brain barrier in injured brain. Furthermore, our current results have the potential to provide a novel insight for other ongoing clinical trials which make use of MSC transplantation aiming to suppress excess inflammation, as well as other diseases such as COVID-19 (Coronavirus Disease 2019).

Significance Statement

We have demonstrated that gap junction mediated direct cell-cell interaction between transplanted mesenchymal stem cells (MSC) and cells of a recipient, including endothelial cell and circulating lymphocyte/monocyte, is one of the prominent pathways that suppress excessive inflammation following MSC transplantation. Our current results have the potential to provide novel insights in clinical trials that make use of MSC transplantation aiming to suppress excess inflammation. This is also relevant for other diseases including COVID-19 (Coronavirus Disease 2019) which can cause fatal multiple organ failure due to vascular deterioration and cytokine storm.

Introduction

Mesenchymal stem cells (MSC), bone marrow mononuclear cells (BM-MNC) and neural stem cells are the major cell sources in cell-based therapies for stroke.[1] However, the therapeutic mechanism of each therapy has been difficult to elucidate and this is likely to be a consequence of their complex composition. Therefore, significant research is underway to generate the next generation of cell-based therapies with improved characteristics. Recently, we demonstrated that direct cell-cell interaction between transplanted BM-MNC and cerebral endothelial cells via gap junction following cell transplantation is the prominent pathway for activation of regenerative processes after ischemia.[2] Our findings revealed that BM-MNC activate injured endothelial cells by providing glucose via gap junction and accelerate VEGF uptake into endothelial cells followed by activation of angiogenesis at post stroke brain.[2]

Similar to BM-MNC transplantation in stroke, a number of clinical trials of MSC transplantation in stroke are ongoing[3] and multiple therapeutic mechanisms have been proposed, including the acceleration of angiogenesis,[4] secretion of multiple cytokines and immunomodulation.[5] However, the significance of the proposed mechanisms have largely been unclear. In this article, we report that MSC receive glucose from endothelial cells via gap junction and suppress VEGF uptake into endothelial cells followed by stabilization of the blood brain barrier in ischemic brain. This finding is essentially the converse of what was expected

with regards to cell-cell interaction between BM-MNC and endothelial cell.

Materials and Methods

The following study was approved by the Animal Care and Use Committee of Institute of Biomedical Research and Innovation and complies with the Guide for the Care and Use of Animals published by the Ministry of Education, Culture, Sports, Science and Technology in Japan. Experiments and results are reported according to the ARRIVE guidelines. A detailed methodological description is available in the supplement.

Preparation of murine mesenchymal stem cells (MSC)

Murine MSC obtained from C57BL/6 mice were purchased from Cyagen Biosciences (CA, USA). MSC were cultured with growth medium (OriCell Mouse MSC Growth Medium; Cyagen Biosciences) according to the manufacturer's protocol. After thawing the freezing ampule, cold growth medium was added, and the cell suspension was centrifuged and the supernatant removed. The re-suspended cells in growth medium were seeded into a flask and incubated at 37ºC and 5% CO_2. After reaching 80-90% confluence, cells were dissociated with 0.25% Trypsin-EDTA (Thermo Fisher, MA, USA) and expanded. The growth medium was changed every three days. Cells in passage 9 were used for *in vitro* and *in vivo* experiments.

Human vascular endothelial growth factor (hVEGF) uptake into human umbilical vein

endothelial cell (HUVEC)

Human umbilical vein endothelial cells (HUVEC, Kurabo, Osaka, Japan) were cultured with medium, serum and growth factors (HuMedia-EB2, Kurabo) according to manufacturer's protocol. HUVEC in passage 6 were used for all experiments. VEGF uptake was evaluated the methods as described elsewhere.[2] Biotin-conjugated hVEGF (R&D Systems, Minneapolis, MN, USA) was incubated with streptavidin-conjugated APC (Thermo Fisher), at a 4:1 molar ratio for 10 minutes at room temperature. HUVEC were harvested and suspended in PBS containing 1% fetal bovine serum (FBS, Thermo Fisher). MSC ($1x10^5$ cells) and APC-labeled hVEGF (10 nM) was added to $1x10^5$ HUVEC, and incubated for 3 hours at 37ºC. After co-incubation, cells were washed twice with PBS and stained with PE-conjugated anti-human CD31 antibody (BD Bioscience, MA, USA), FITC-conjugated anti-mouse Sca1 antibody (BD Bioscience, NJ, USA), and 7-AAD (BD Bioscience). The level of APC in HUVEC (CD31-positive, Sca1-negative and 7AAD-negative) was evaluated using a FACS Calibur fluorescent cell sorter (BD Bioscience). To evaluate the relevance of gap junction mediated cell-cell interaction between HUVEC and MSC, MSC were incubated with the gap junction uncoupling agent 1-octanol (1 mM; Merck, NJ, USA) or gap junction-blocking carbenoxolone (CBX: 100 µM; Sigma, MO, USA) for 10 minutes, and then washed twice with HuMedia-EB2 before co-culturing with HUVEC.

Glucose concentration measurements

The glucose concentrations in MSC and HUVEC were measured using a glucose assay kit (Biovision, CA, USA) according to manufacturer's protocol. Briefly, $2x10^5$ MSC or $2x10^5$ HUVEC were incubated with HuMedia-EB2 (0.1% glucose) for 1 hour and washed twice with PBS before cell lysis. The glucose and protein concentration in cell lysates was evaluated by glucose assay kit.

Glucose homologue transfer between HUVEC and MSC

HUVEC and MSC were incubated with 25 μM 2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; Peptide Institute, Osaka, Japan) in HuMedia-EB2 without glucose (Kurabo) for 1 hour at 37ºC. After washing the cells twice with PBS, $1x10^5$ of HUVEC or MSC were co-cultured in 100 μl PBS for 3 hours at 37ºC. Mean fluorescence intensity in each cell type were measured using FACS. For control, $1x10^5$ HUVEC or $1x10^5$ MSC alone were cultured in 100 μl PBS for 3 hours at 37ºC. HUVEC and MSC without 2-NBDG were also prepared as 2-NBDG negative controls.

Loading of low molecular weight fluorescence molecules in cytoplasm of MSC or white blood cells (WBC)

MSC were incubated with 5 μM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Dojindo, Kumamoto Japan) for 30 minutes at 37ºC according to manufacturer's protocol. BCECF-AM is converted to 2',7'-bis-(2-carboxyethyl)-

5-(6)-carboxyfluorescein (BCECF) in the cytoplasm and BCECF-loaded MSC were washed twice with PBS before use for *in vitro* and *in vivo* experiments. Murine blood samples were obtained following puncture of the left ventricle of 9 week old male C57BL/6 mice (Clea Japan, Tokyo, Japan) with heparin sodium 50 U/mL (Mochida Pharmaceutical, Tokyo, Japan) and red blood cells were lysed with red blood cell lysis buffer (BD Bioscience) according to the manufacture's protocol. BCECF-loaded WBC was prepared by the same way with MSC. $1x10^6$ BCECF-loaded WBC were incubated with $1x10^5$ BCECF-unloaded MSC for 2 hours and the transfer of BCECF from WBC to MSC via gap junction was evaluated by FACS[2] without or with blockade of gap junction of MSC by 1-octanol as we described above. Cell populations (lymphocytes, monocytes and granulocytes) were characterized using FACS as described previously.[6]

Induction of focal cerebral ischemia and injection of MSC

A murine stroke model with excellent reproducibility that made use of 7-week-old male CB-17 mice (C.B-17/Icr- +/+Jcl: Oriental yeast, Tokyo, Japan) was utilized as described previously.[7] Briefly, permanent focal cerebral ischemia was induced by permanent ligation and disconnection of the distal portion of the left middle cerebral artery (MCA) using bipolar forceps under 3% halothane inhalation anesthesia. During surgery, rectal temperature was monitored and controlled at 37.0±0.2ºC by a feedback-regulated heating pad. Cerebral blood

flow (CBF) in the MCA area was also monitored. Mice showing a ≥75% decrease in CBF immediately after MCA occlusion were used for *in vivo* experiments (success rate was 100%). The weight of animals was ~20-25 grams before surgery. Twenty four hours after induction of stroke, $5x10^5$ MSC or heparinized HBSS were injected via a tail vein.

Immunohistochemistry

Mice were anesthetized using sodium pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde (PFA). The brain was carefully removed and cut into coronal sections (20 μm) using a vibratome (Leica, Wetzlar, Germany). Sections were immunostained with primary antibodies against CD31 (BD Pharmingen, CA, USA; 1:50), connexin 37 (Cloud-Clone, TX, USA; 1:50), connexin 43 (Proteintech, IL, USA; 1:200) or DAPI (Thermo Fisher, 1:1,000). Alexa 555-coupled antibody (Novus Biologicals, CO, USA) was used as the secondary antibody to visualize CD31. Alexa 647 (Novus Biologicals)-coupled secondary antibodies were used to detect connexin 37 or connexin 43 antibody. Anti-F4/80 (Serotec NC, USA; 1:50), was visualized by the 3,3'-diaminobenzidine (DAB) method and counterstained with Mayer's Hematoxylin Solution (Wako, Osaka, Japan). The number of $F4/80^+$ cells in $0.25mm^2$ were counted in a blinded manner.

For *in vitro* analysis of BCECF transfer from MSC to HUVEC, BCECF-loaded MSC ($1x10^4$ cells) were co-cultured with HUVEC ($1x10^5$ cells) for 1 hour. After co-culture, cells were fixed

with 4% PFA for 15 minutes and stained with anti-Sca-1 antibody (BD Pharmingen, 1:50) and vWF (Merck 1:500) for identification of MSC and HUVEC respectively.

Data analysis

Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA). Where indicated, individual comparisons were performed using Student's t-test. In all experiments, the mean ± SD are reported.

Results

MSC suppress VEGF uptake into HUVEC through gap junction mediated cell-cell interaction

VEGF is one of the most prominent pro-angiogenic factors.[2] Cerebral endothelial cells are known to uptake VEGF followed by activation of angiogenesis with increased permeability of blood brain barrier.[8] We previously showed that BM-MNC increase VEGF uptake into HUVEC via gap junction mediated cell-cell interaction.[2] To investigate the analogous property of MSC, these were co-cultured with HUVEC and any changes in VEGF uptake into HUVEC were assessed. Surprisingly, a significant reduction of VEGF uptake into HUVEC was observed when co-cultured with MSC (Figure 1A). To evaluate the importance of gap junction channel of MSC, its 1-octanol or carbenoxolone[9] mediated blockade was investigated. Our results show

that the blockade of gap junction of MSC are abolished when co-incubated with HUVEC. These findings indicate that MSC reduce VEGF uptake into HUVEC by gap junction-mediated cell-cell interaction, in contrast to BM-MNC that increase VEGF uptake into HUVEC.

Glucose concentration in MSC and HUVEC

We previously showed that the glucose concentration in BM-MNC is significantly higher than in HUVEC and that the transfer of glucose from BM-MNC to endothelial cells is one of the triggers that activates angiogenesis in endothelial cells. To investigate the analogous property of MSC, the concentration of glucose in MSC and endothelial cells was compared *in vitro*. In marked contrast to our expectation, MSC were shown to contain approximately half the concentration of glucose when compared with HUVEC (Figure 1B). The transfer of uptaken glucose between HUVEC and MSC was evaluated using the fluorescence-positive glucose homologue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG)[2]. MSC and HUVEC was separately incubated with 2-NBDG and washed twice before co-culture. A significant decrease in 2-NBDG levels was observed in HUVEC when co-cultured with MSC (Figure 1C). To confirm the importance of gap junction mediated cell-cell interaction between HUVEC and MSC, the gap junction channel of MSC was blocked by 1-octanol or carbenoxolone, and co-incubated with HUVEC. As expected, blockade of the gap junction channel of MSC resulted in decreased 2-NBDG levels in these cells. In contrast, the levels of

2-NBDG in MSC were significantly increased when co-cultured with HUVEC (Figure 1D). These findings indicate that the glucose concentration in MSC is reduced, which contrasts to that of BM-MNC that increases glucose concentration of HUVEC via gap junction mediated cell-cell small molecule transfer.

In vivo cell-cell interaction between transplanted MSC and endothelial cells through gap junction

In our previous study, we demonstrated *in vivo* cell-cell interaction between transplanted BM-MNC and endothelial cells through gap junction using BCECF-loaded BM-MNC[2]. To investigate the analogous property of MSC, BCECF-loaded MSC were transplanted intravenously into mice at 24 hours after MCA occlusion. Mice were sacrificed 10 minutes after MSC transplantation, and co-localization of gap junctions and transferred BCECF in endothelial cells was evaluated by fluorescence confocal microscopy. Transferred BCECF-positive signals were observed in endothelial cells at 10 minutes after cell transplantation, mostly overlapping with connexin 37 or 43 (Figure 2A-D). As in the case of BM-MNC transplantation following stroke,[2] transplanted MSC nor obstruction of cerebral vasculature by transplanted MSC were rarely observed in the stroke area. It is noteworthy that the expression of connexin 37 or 43 were observed in the cell membrane of endothelial cells which are adjacent to the transferred BCECF signal at nuclei/cytosol of the cells. These findings indicate direct

cell-cell interaction via gap junction between MSC and endothelial cells *in vivo*, and are analogous to the cell-cell interaction between BM-MNC and endothelial cells following stroke. To confirm BCECF-positive signal transfer from MSC to endothelial cells *in vitro*, BCECF-loaded MSC were cultured with HUVEC and BCECF-positive HUVEC were observed (Figure 2E).

Blockade of gap junction channels of transplanted MSC abolishes anti-inflammatory effects of MSC in mice brain

MSC are known to suppress inflammatory reactions following stroke, including macrophage/microglia activation in the affected brain.[5] However, the mechanism of inflammatory suppression by MSC transplantation in ischemic brain has been disputed. To evaluate the contribution of gap junction mediated cell-cell interaction between transplanted MSC and endothelial cells on suppression of inflammatory reactions following stroke, the effect of MSC with or without gap junction channel blockade was investigated. The results show a significant reduction of macrophage/microglia activation (number of $F4/80^+$ cells) at 72 hours after induction of stroke (Figure 3A), although the anti-inflammatory effect was absent when MSC were pre-treated with the gap junction blocker prior to transplantation (Figure 3B). These findings indicate the significance of the gap junction mediated cell-cell interaction between MSC and endothelial cells *in vivo* to suppress inflammatory reactions following stroke.

244 **Cell-cell interaction between MSC and circulating WBC**

245 Circulating WBC are also known to express gap junction.[10] To investigate direct cell-cell
246 interaction between transplanted MSC and circulating WBC, transfer of small molecules from
247 WBC to MSC was evaluated *in vitro*. As shown in Figure 4A, transfer of BCECF from WBC
248 to MSC was observed and the transfer was inhibited by the blockade of gap junction. Analysis
249 of WBC with or without co-culture with MSC revealed the level of BCECF was significantly
250 decreased in lymphocyte and monocyte by co-culture with MSC (Figure 4B). These findings
251 indicated the transplanted MSC affect circulating WBS along with injured endothelial cells in
252 brain and major WBC that react with MSC are lymphocyte and monocyte.

253

254 **Discussion**

255 We have demonstrated that MSC decreases VEGF uptake into endothelial cells *in vitro* and
256 suppress inflammation *in vivo* through gap junction mediated cell-cell interaction. Our findings
257 indicate that gap junction-mediated signaling is one of the major pathways for MSC-mediated
258 suppression of inflammation in the brain following stroke.

259 A gap junction channel between two cells is composed of two connexin in each half of the cell
260 pair and they allow the prompt movement of small molecules according to their concentration
261 gradient between cells.[11] In our previous study, we had shown BM-MNC increases VEGF

uptake into endothelial cells via gap junction mediated cell-cell interaction and transfer of glucose from BM-MNC to endothelial cells via gap junction triggers angiogenesis.[2] Since one of the proposed therapeutic mechanisms of MSC transplantation for stroke had been acceleration of angiogenesis,[4] we expected a similar therapeutic mechanism in MSC with BM-MNC. However, the transfer of glucose from endothelial cells to MSC was observed in this study with decreased VEGF uptake into endothelial cells by gap junction-mediated cell-cell interaction. These findings indicate that the therapeutic mechanisms of MSC and BM-MNC would be significantly different (Figure 5A-B).

Uptake of VEGF is one of the key signals for endothelial cells to activate angiogenesis and increase permeability of barrier function following increased inflammation in ischemic brain.[12-14] In contrast, suppression of VEGF uptake attenuates blood-brain barrier disruption.[15] We have demonstrated that MSC suppressed VEGF uptake of endothelial cells *in vitro* and intravenous injection of MSC reduced inflammatory responses at peri-stroke area. Furthermore, inhibition of gap junction channel of MSC abolishes the effect of MSC *in vitro* and *in vivo*. These results indicate that transplanted MSC suppressed inflammatory response in brain by inhibiting VEGF uptake into cerebral endothelial cells via gap junction mediated cell-cell interaction.

Pericytes are known to be one of the MSC[16] that are important for the stabilization of the blood-brain barrier.[17] Pericytes and cerebral endothelial cells are connected via gap junction and their

dissociation after ischemia had been shown to increase permeability followed by activation of macrophages.[17] These findings suggest that transplanted MSC can substitute the function of dissociated pericytes.

We have demonstrated that small molecules can be transferred from circulating WBC to MSC via gap junction (Figure 5C). Our results also indicate that the major cell populations that react with MSC are lymphocytes and monocytes, but not granulocytes. Lymphocyte causes graft-versus-host disease (GvHD) after allogenic hematopoietic stem cell transplantation[18] and MSC transplantation is known to have a therapeutic effect in GvHD, although the mechanism is not fully understood.[19] Activation of lymphocytes are known to be related to increased glucose level in lymphocytes[20] and glucose is one of the major factors that are transferred via gap junction between cells.[2] Our current results relating to the small molecule outflow from lymphocytes to MSC via gap junction provides a novel insight of MSC therapy for GvHD. MSC transplantation is also known to have a therapeutic potential for COVID-19, although this mechanism is not fully understood either.[21] Fatal vascular deterioration caused by cytokine release syndrome has been shown to be critical COVID-19 patients[22] and the major players of cytokine release syndrome caused by COVID-19 are monocytes, lymphocytes and endothelial cells.[23] Our current data indicate that MSC have the potential to directly regulate monocyte, lymphocyte and endothelial cells via gap junction mediated cell-cell interaction. Although further studies

are required to reveal the full linkage between MSC, monocytes, lymphocytes and endothelial cells via gap junction mediated cell-cell interaction, extending our hypothesis to COVID-19, although speculative at present has the potential to provide a novel insight for new therapeutic strategy against the COVID-19 cytokine storm.

In conclusion, our findings establish that gap junction mediated direct cell-cell interaction between transplanted MSC and cells of recipient, including endothelial cells and circulating lymphocyte/monocyte is highly significant. Furthermore, our current results have potential to provide a novel insight to other clinical trials that make use of MSC transplantation aiming to suppress excess inflammation, which are ongoing for various diseases including COVID-19.[24]

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Disclosures

Dr. Taguchi reports grants from Kaneka and Mitsubishi Tanabe Pharma outside the submitted work; in addition, he has a patent to patent pending (PCT/JP2019/008701).

316 **References**

317 1. Janowski M, Wagner DC, Boltze J. Stem cell-based tissue replacement after stroke:
318 Factual necessity or notorious fiction? *Stroke*. 2015;46:2354-2363

319 2. Kikuchi-Taura A, Okinaka Y, Takeuchi Y, et al. Bone marrow mononuclear cells activate
320 angiogenesis via gap junction-mediated cell-cell interaction. *Stroke*. 2020;51:1279-
321 1289

322 3. Borlongan CV. Concise review: Stem cell therapy for stroke patients: Are we there yet?
323 *Stem Cells Transl Med*. 2019;8:983-988

324 4. Maria Ferri AL, Bersano A, Lisini D, et al. Mesenchymal stem cells for ischemic stroke:
325 Progress and possibilities. *Curr Med Chem*. 2016;23:1598-1608

326 5. Alessandrini M, Preynat-Seauve O, De Bruin K, et al. Stem cell therapy for neurological
327 disorders. *S Afr Med J*. 2019;109:70-77

328 6. Melgert BN, Spaans F, Borghuis T, et al. Pregnancy and preeclampsia affect monocyte
329 subsets in humans and rats. *PLoS One*. 2012;7:e45229

330 7. Taguchi A, Kasahara Y, Nakagomi T, et al. A reproducible and simple model of
331 permanent cerebral ischemia in cb-17 and scid mice. *J Exp Stroke Transl Med*.
332 2010;3:28-33

333 8. Shim JW, Madsen JR. Vegf signaling in neurological disorders. *Int J Mol Sci*. 2018;19

9. Nielsen MS, Axelsen LN, Sorgen PL, et al. Gap junctions. *Compr Physiol*. 2012;2:1981-2035

10. Ni X, Li XZ, Fan ZR, et al. Increased expression and functionality of the gap junction in peripheral blood lymphocytes is associated with hypertension-mediated inflammation in spontaneously hypertensive rats. *Cell Mol Biol Lett*. 2018;23:40

11. Kumar NM, Gilula NB. The gap junction communication channel. *Cell*. 1996;84:381-388

12. Hudson N, Powner MB, Sarker MH, et al. Differential apicobasal vegf signaling at vascular blood-neural barriers. *Dev Cell*. 2014;30:541-552

13. Weis SM, Cheresh DA. Pathophysiological consequences of vegf-induced vascular permeability. *Nature*. 2005;437:497-504

14. Croll SD, Ransohoff RM, Cai N, et al. Vegf-mediated inflammation precedes angiogenesis in adult brain. *Exp Neurol*. 2004;187:388-402

15. Zhang HT, Zhang P, Gao Y, et al. Early vegf inhibition attenuates blood-brain barrier disruption in ischemic rat brains by regulating the expression of mmps. *Mol Med Rep*. 2017;15:57-64

16. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*. 2006;119:2204-2213

17. Keaney J, Campbell M. The dynamic blood-brain barrier. *FEBS J*. 2015;282:4067-4079

18. Ramachandran V, Kolli SS, Strowd LC. Review of graft-versus-host disease. *Dermatol Clin*. 2019;37:569-582

19. Gao F, Chiu SM, Motan DA, et al. Mesenchymal stem cells and immunomodulation: Current status and future prospects. *Cell Death Dis*. 2016;7:e2062

20. Macintyre AN, Gerriets VA, Nichols AG, et al. The glucose transporter glut1 is selectively essential for cd4 t cell activation and effector function. *Cell Metab*. 2014;20:61-72

21. Golchin A, Seyedjafari E, Ardeshirylajimi A. Mesenchymal stem cell therapy for covid-19: Present or future. *Stem Cell Rev Rep*. 2020;16:427-433

22. von der Thusen J, van der Eerden M. Histopathology and genetic susceptibility in covid-19 pneumonia. *Eur J Clin Invest*. 2020;50:e13259

23. Moore JB, June CH. Cytokine release syndrome in severe covid-19. *Science*. 2020;368:473-474

24. Chrzanowski W, Kim SY, McClements L. Can stem cells beat covid-19: Advancing stem cells and extracellular vesicles toward mainstream medicine for lung injuries associated with sars-cov-2 infections. *Front Bioeng Biotechnol*. 2020;8:554

Figure Legends

Figure 1. Decreased hVEGF uptake into HUVEC when co-cultured with MSC and glucose transfer between these cells

(A) APC-labelled VEGF uptake in HUVEC was evaluated using FACS. The percentage of APC-positive HUVEC was significantly decreased when co-cultured with MSC. Blockade of gap junction of MSC by 1-octanol (OCT) or carbenoxolone (CBX) abolishes this effect. (B) Glucose concentration in HUVEC was higher than that in MSC in the steady state. (C) Decreased 2-NBDG levels was observed in HUVEC during co-culture with MSC. It is noteworthy that blockade of gap junction of MSC by 1-octanol (OCT) or carbenoxolone (CBX) abolishes this effect. (D) In contrast, the level of 2-NBDG in MSC was increased when co-cultured with HUVEC. Blockade of gap junction of MSC by 1-octanol (OCT) or carbenoxolone (CBX) abolishes this effect. $^{**}p<0.01$, (A, D), $^{*}p<0.05$ (B, C). n=3 in each group (A-D).

Figure 2. Localization of gap junction and transfer of BCECF in endothelial cells

(A,B) Co-localization of connexin 37 (light blue) and transferred BCECF (green) was observed in cerebral endothelial cells (red) (A). Merged image with explanation (B). (C, D) Co-localization of connexin 43 (light blue) and transferred BCECF (green) in endothelial cells (red) (C). Merged image with explanation (D). (E) After co-incubation with BCECF-loaded Sca-1

positive MSC (red), transferred BCECF (green) was observed at vWF-positive HUVEC (light blue). Scale bars: 5 μm (A,C), 1μm (B,D), 100μm (E). Arrow heads indicate vWF-positive HUVEC (E).

Figure 3. MSC suppress excess inflammation following stroke

(A) Representative images of $F4/80^+$ at 72 hours following stroke. MSC transplantation significantly suppressed the number of $F4/80^+$ cells in the lesion border zone. (B) Application of a gap junction blocker (1-octanol) reduced the anti-inflammatory effect of MSC. *p<0.01 vs control, N=6 in each group (A, B).

Figure 4. Gap junction mediated cell-cell interaction between circulating WBC and MSC

(A) FACS analysis reveals transfer of low molecular weight substance (BCECF) from WBC to MSC. The transfer was inhibited by the blockade of gap junction of MSC by 1-octanol. (B) The level of BCECF was significantly decreased in monocytes and lymphocytes when co-incubated with MSC. **p<0.01 vs control (A). *p<0.05 and **p<0.01 *vs* MSC(-) control (B). n=4 (A) and n=3 (B) in each group.

Figure 5. Schematic representation of our conclusions

(A) We have previously demonstrated that BM-MNC, which have a higher glucose concentration than endothelial cells, supply glucose to endothelia cell via gap junction followed by increased uptake of VEGF into endothelial cell with activation of angiogenesis. (B) In marked contrast, MSC have lower glucose concentration than endothelial cells. The reverse glucose flow suppresses VEGF uptake into endothelial cell followed by reducing the blood brain barrier permeability. (C) In addition to the interaction between MSC and endothelial cells, direct cell-cell interaction via gap junction was observed between MSC and circulating lymphocytes/monocytes.