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

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

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33
34 Key words: mesenchymal stem cell; cell therapy; immunosuppression; gap junction; COVID-
35 19.

36 **Abstract**

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

53

54 **Significance Statement**

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

63

64 **Introduction**

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

75 Similar to BM-MNC transplantation in stroke, a number of clinical trials of MSC
76 transplantation in stroke are ongoing³ and multiple therapeutic mechanisms have been proposed,
77 including the acceleration of angiogenesis,⁴ secretion of multiple cytokines and
78 immunomodulation.⁵ However, the significance of the proposed mechanisms have largely been
79 unclear. In this article, we report that MSC receive glucose from endothelial cells via gap
80 junction and suppress VEGF uptake into endothelial cells followed by stabilization of the blood
81 brain barrier in ischemic brain. This finding is essentially the converse of what was expected

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

83

84 **Materials and Methods**

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

90 **Preparation of murine mesenchymal stem cells (MSC)**

91 Murine MSC obtained from C57BL/6 mice were purchased from Cyagen Biosciences (CA,
92 USA). MSC were cultured with growth medium (OriCell Mouse MSC Growth Medium;
93 Cyagen Biosciences) according to the manufacturer's protocol. After thawing the freezing
94 ampule, cold growth medium was added, and the cell suspension was centrifuged and the
95 supernatant removed. The re-suspended cells in growth medium were seeded into a flask and
96 incubated at 37°C and 5% CO₂. After reaching 80-90% confluence, cells were dissociated with
97 0.25% Trypsin-EDTA (Thermo Fisher, MA, USA) and expanded. The growth medium was
98 changed every three days. Cells in passage 9 were used for *in vitro* and *in vivo* experiments.

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

100 **endothelial cell (HUVEC)**

101 Human umbilical vein endothelial cells (HUVEC, Kurabo, Osaka, Japan) were cultured with
102 medium, serum and growth factors (HuMedia-EB2, Kurabo) according to manufacturer's
103 protocol. HUVEC in passage 6 were used for all experiments. VEGF uptake was evaluated the
104 methods as described elsewhere.² Biotin-conjugated hVEGF (R&D Systems, Minneapolis, MN,
105 USA) was incubated with streptavidin-conjugated APC (Thermo Fisher), at a 4:1 molar ratio
106 for 10 minutes at room temperature. HUVEC were harvested and suspended in PBS containing
107 1% fetal bovine serum (FBS, Thermo Fisher). MSC (1×10^5 cells) and APC-labeled hVEGF (10
108 nM) was added to 1×10^5 HUVEC, and incubated for 3 hours at 37°C. After co-incubation, cells
109 were washed twice with PBS and stained with PE-conjugated anti-human CD31 antibody (BD
110 Bioscience, MA, USA), FITC-conjugated anti-mouse Sca1 antibody (BD Bioscience, NJ, USA),
111 and 7-AAD (BD Bioscience). The level of APC in HUVEC (CD31-positive, Sca1-negative and
112 7AAD-negative) was evaluated using a FACS Calibur fluorescent cell sorter (BD Bioscience).
113 To evaluate the relevance of gap junction mediated cell-cell interaction between HUVEC and
114 MSC, MSC were incubated with the gap junction uncoupling agent 1-octanol (1 mM; Merck,
115 NJ, USA) or gap junction-blocking carbenoxolone (CBX: 100 μ M; Sigma, MO, USA) for 10
116 minutes, and then washed twice with HuMedia-EB2 before co-culturing with HUVEC.

117 **Glucose concentration measurements**

118 The glucose concentrations in MSC and HUVEC were measured using a glucose assay kit
119 (Biovision, CA, USA) according to manufacturer's protocol. Briefly, 2×10^5 MSC or 2×10^5
120 HUVEC were incubated with HuMedia-EB2 (0.1% glucose) for 1 hour and washed twice with
121 PBS before cell lysis. The glucose and protein concentration in cell lysates was evaluated by
122 glucose assay kit.

123 **Glucose homologue transfer between HUVEC and MSC**

124 HUVEC and MSC were incubated with $25 \mu\text{M}$ 2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-
125 2-deoxyglucose (2-NBDG; Peptide Institute, Osaka, Japan) in HuMedia-EB2 without glucose
126 (Kurabo) for 1 hour at 37°C . After washing the cells twice with PBS, 1×10^5 of HUVEC or MSC
127 were co-cultured in $100 \mu\text{l}$ PBS for 3 hours at 37°C . Mean fluorescence intensity in each cell
128 type were measured using FACS. For control, 1×10^5 HUVEC or 1×10^5 MSC alone were
129 cultured in $100 \mu\text{l}$ PBS for 3 hours at 37°C . HUVEC and MSC without 2-NBDG were also
130 prepared as 2-NBDG negative controls.

131 **Loading of low molecular weight fluorescence molecules in cytoplasm of MSC or white** 132 **blood cells (WBC)**

133 MSC were incubated with $5 \mu\text{M}$ 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein,
134 acetoxymethyl ester (BCECF-AM; Dojindo, Kumamoto Japan) for 30 minutes at 37°C
135 according to manufacturer's protocol. BCECF-AM is converted to 2',7'-bis-(2-carboxyethyl)-

136 5-(6)-carboxyfluorescein (BCECF) in the cytoplasm and BCECF-loaded MSC were washed
137 twice with PBS before use for *in vitro* and *in vivo* experiments. Murine blood samples were
138 obtained following puncture of the left ventricle of 9 week old male C57BL/6 mice (Clea Japan,
139 Tokyo, Japan) with heparin sodium 50 U/mL (Mochida Pharmaceutical, Tokyo, Japan) and red
140 blood cells were lysed with red blood cell lysis buffer (BD Bioscience) according to the
141 manufacture's protocol. BCECF-loaded WBC was prepared by the same way with MSC. 1×10^6
142 BCECF-loaded WBC were incubated with 1×10^5 BCECF-unloaded MSC for 2 hours and the
143 transfer of BCECF from WBC to MSC via gap junction was evaluated by FACS² without or
144 with blockade of gap junction of MSC by 1-octanol as we described above. **Cell populations**
145 **(lymphocytes, monocytes and granulocytes) were characterized using FACS as described**
146 **previously.⁶**

147 **Induction of focal cerebral ischemia and injection of MSC**

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

154 flow (CBF) in the MCA area was also monitored. Mice showing a $\geq 75\%$ decrease in CBF
155 immediately after MCA occlusion were used for *in vivo* experiments (success rate was 100%).
156 The weight of animals was ~20-25 grams before surgery. Twenty four hours after induction of
157 stroke, 5×10^5 MSC or heparinized HBSS were injected via a tail vein.

158 **Immunohistochemistry**

159 Mice were anesthetized using sodium pentobarbital and perfused transcardially with saline
160 followed by 4% paraformaldehyde (PFA). The brain was carefully removed and cut into coronal
161 sections (20 μm) using a vibratome (Leica, Wetzlar, Germany). Sections were immunostained
162 with primary antibodies against CD31 (BD Pharmingen, CA, USA; 1:50), connexin 37 (Cloud-
163 Clone, TX, USA; 1:50), connexin 43 (Proteintech, IL, USA; 1:200) or DAPI (Thermo Fisher,
164 1:1,000). Alexa 555-coupled antibody (Novus Biologicals, CO, USA) was used as the
165 secondary antibody to visualize CD31. Alexa 647 (Novus Biologicals)-coupled secondary
166 antibodies were used to detect connexin 37 or connexin 43 antibody. Anti-F4/80 (Serotec NC,
167 USA; 1:50), was visualized by the 3,3'-diaminobenzidine (DAB) method and counterstained
168 with Mayer's Hematoxylin Solution (Wako, Osaka, Japan). The number of F4/80⁺ cells in
169 0.25mm^2 were counted in a blinded manner.

170 *For in vitro* analysis of BCECF transfer from MSC to HUVEC, BCECF-loaded MSC (1×10^4
171 cells) were co-cultured with HUVEC (1×10^5 cells) for 1 hour. After co-culture, cells were fixed

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

174 **Data analysis**

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

178

179 **Results**

180 **MSC suppress VEGF uptake into HUVEC through gap junction mediated cell-cell** 181 **interaction**

182 VEGF is one of the most prominent pro-angiogenic factors.² Cerebral endothelial cells are
183 known to uptake VEGF followed by activation of angiogenesis with increased permeability of
184 blood brain barrier.⁸ We previously showed that BM-MNC increase VEGF uptake into HUVEC
185 via gap junction mediated cell-cell interaction.² To investigate the analogous property of MSC,
186 these were co-cultured with HUVEC and any changes in VEGF uptake into HUVEC were
187 assessed. Surprisingly, a significant reduction of VEGF uptake into HUVEC was observed
188 when co-cultured with MSC (Figure 1A). To evaluate the importance of gap junction channel
189 of MSC, its 1-octanol or carbenoxolone⁹ mediated blockade was investigated. Our results show

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

193 **Glucose concentration in MSC and HUVEC**

194 We previously showed that the glucose concentration in BM-MNC is significantly higher than
195 in HUVEC and that the transfer of glucose from BM-MNC to endothelial cells is one of the
196 triggers that activates angiogenesis in endothelial cells. To investigate the analogous property
197 of MSC, the concentration of glucose in MSC and endothelial cells was compared *in vitro*. In
198 marked contrast to our expectation, MSC were shown to contain approximately half the
199 concentration of glucose when compared with HUVEC (Figure 1B). The transfer of uptaken
200 glucose between HUVEC and MSC was evaluated using the fluorescence-positive glucose
201 homologue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG)².

202 MSC and HUVEC was separately incubated with 2-NBDG and washed twice before co-culture.
203 A significant decrease in 2-NBDG levels was observed in HUVEC when co-cultured with MSC
204 (Figure 1C). To confirm the importance of gap junction mediated cell-cell interaction between
205 HUVEC and MSC, the gap junction channel of MSC was blocked by 1-octanol or
206 carbenoxolone, and co-incubated with HUVEC. As expected, blockade of the gap junction
207 channel of MSC resulted in decreased 2-NBDG levels in these cells. In contrast, the levels of

208 2-NBDG in MSC were significantly increased when co-cultured with HUVEC (Figure 1D).

209 These findings indicate that the glucose concentration in MSC is reduced, which contrasts to
210 that of BM-MNC that increases glucose concentration of HUVEC via gap junction mediated
211 cell-cell small molecule transfer.

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

214 In our previous study, we demonstrated *in vivo* cell-cell interaction between transplanted BM-
215 MNC and endothelial cells through gap junction using BCECF-loaded BM-MNC². To
216 investigate the analogous property of MSC, BCECF-loaded MSC were transplanted
217 intravenously into mice at 24 hours after MCA occlusion. Mice were sacrificed 10 minutes after
218 MSC transplantation, and co-localization of gap junctions and transferred BCECF in
219 endothelial cells was evaluated by fluorescence confocal microscopy. Transferred BCECF-
220 positive signals were observed in endothelial cells at 10 minutes after cell transplantation,
221 mostly overlapping with connexin 37 or 43 (Figure 2A-D). As in the case of BM-MNC
222 transplantation following stroke,² transplanted MSC nor obstruction of cerebral vasculature by
223 transplanted MSC were rarely observed in the stroke area. It is noteworthy that the expression
224 of connexin 37 or 43 were observed in the cell membrane of endothelial cells which are adjacent
225 to the transferred BCECF signal at nuclei/cytosol of the cells. These findings indicate direct

226 cell-cell interaction via gap junction between MSC and endothelial cells *in vivo*, and are
227 analogous to the cell-cell interaction between BM-MNC and endothelial cells following stroke.
228 **To confirm BCECF-positive signal transfer from MSC to endothelial cells *in vitro*, BCECF-**
229 **loaded MSC were cultured with HUVEC and BCECF-positive HUVEC were observed (Figure**
230 **2E).**

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

233 MSC are known to suppress inflammatory reactions following stroke, including
234 macrophage/microglia activation in the affected brain.⁵ However, the mechanism of
235 inflammatory suppression by MSC transplantation in ischemic brain has been disputed. To
236 evaluate the contribution of gap junction mediated cell-cell interaction between transplanted
237 MSC and endothelial cells on suppression of inflammatory reactions following stroke, the effect
238 of MSC with or without gap junction channel blockade was investigated. The results show a
239 significant reduction of macrophage/microglia activation (number of F4/80⁺ cells) at 72 hours
240 after induction of stroke (Figure 3A), although the anti-inflammatory effect was absent when
241 MSC were pre-treated with the gap junction blocker prior to transplantation (Figure 3B). These
242 findings indicate the significance of the gap junction mediated cell-cell interaction between
243 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

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

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

278 Pericytes are known to be one of the MSC¹⁶ that are important for the stabilization of the blood-
279 brain barrier.¹⁷ Pericytes and cerebral endothelial cells are connected via gap junction and their

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

283 We have demonstrated that small molecules can be transferred from circulating WBC to MSC
284 via gap junction (Figure 5C). Our results also indicate that the major cell populations that react
285 with MSC are lymphocytes and monocytes, but not granulocytes. Lymphocyte causes graft-
286 versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation¹⁸ and MSC
287 transplantation is known to have a therapeutic effect in GvHD, although the mechanism is not
288 fully understood.¹⁹ Activation of lymphocytes are known to be related to increased glucose level
289 in lymphocytes²⁰ and glucose is one of the major factors that are transferred via gap junction
290 between cells.² Our current results relating to the small molecule outflow from lymphocytes to
291 MSC via gap junction provides a novel insight of MSC therapy for GvHD. MSC transplantation
292 is also known to have a therapeutic potential for COVID-19, although this mechanism is not
293 fully understood either.²¹ Fatal vascular deterioration caused by cytokine release syndrome has
294 been shown to be critical COVID-19 patients²² and the major players of cytokine release
295 syndrome caused by COVID-19 are monocytes, lymphocytes and endothelial cells.²³ Our
296 current data indicate that MSC have the potential to directly regulate monocyte, lymphocyte
297 and endothelial cells via gap junction mediated cell-cell interaction. Although further studies

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

302 In conclusion, our findings establish that gap junction mediated direct cell-cell interaction
303 between transplanted MSC and cells of recipient, including endothelial cells and circulating
304 lymphocyte/monocyte is highly significant. Furthermore, our current results have potential to
305 provide a novel insight to other clinical trials that make use of MSC transplantation aiming to
306 suppress excess inflammation, which are ongoing for various diseases including COVID-19.²⁴

307

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311

312 **Disclosures**

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

315

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- 369

370 **Figure Legends**

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

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

382

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

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

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

391

392 **Figure 3. MSC suppress excess inflammation following stroke**

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

397

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

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

404

405 **Figure 5. Schematic representation of our conclusions**

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