



Title	Identification of Chemoattractive Factors Involved in the Migration of Bone Marrow-Derived Mesenchymal Stem Cells to Brain Lesions Caused by Prions
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1 Identification of chemoattractive factors involved in the migration of bone marrow-derived
2 mesenchymal stem cells to brain lesions caused by prion

3

4 Running title: Chemotactic factors for MSCs to prion-specific lesions

5

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23

24 **ABSTRACT**

25

26 Bone marrow-derived mesenchymal stem cells (MSCs) have been reported to migrate to
27 brain lesions of neurodegenerative diseases; however, the precise mechanisms by which
28 MSCs migrate remain to be elucidated. In this study, we carried out *in vitro* migration assay
29 to investigate chemoattractive factors for MSCs in the brains of prion-infected mice. The
30 migration of immortalized human MSCs (hMSCs) was reduced by their pre-treatment with
31 antibodies against the chemokine receptors, CCR3, CCR5, CXCR3 and CXCR4 and by
32 pre-treatment of brain extracts of prion-infected mice with antibodies against the
33 corresponding ligands, suggesting the involvement of these receptors and their ligands in the
34 migration of hMSCs. In agreement with the results of an *in vitro* migration assay, hMSCs in
35 the corpus callosum, which are considered to be migrating from the transplanted area towards
36 brain lesions of prion-infected mice, expressed CCR3, CCR5, CXCR3 and CXCR4. The
37 combined *in vitro* and *in vivo* analyses suggest that CCR3, CCR5, CXCR3 and CXCR4 and
38 their corresponding ligands are involved in the migration of hMSCs to the brain lesions
39 caused by prion propagation. Additionally, hMSCs that had migrated to the right
40 hippocampus of prion-infected mice expressed CCR1, CX3CR1 and CXCR4, implying the
41 involvement of these chemokine receptors in hMSC functions after chemotactic migration.
42 Further elucidation of the mechanisms that underlie the migration of MSCs may provide
43 useful information regarding application of MSCs to the treatment of prion diseases.

44

45 **INTRODUCTION**

46

47 Prion diseases are fatal neurodegenerative disorders in humans and animals that are
48 characterized by the accumulation of a disease-specific isoform of the prion protein (PrP^{Sc}),
49 astrocytosis, microglial activation, spongiosis and neuronal cell death in the central nervous
50 system (CNS). Although the etiology of the diseases is not clear, conversion of the normal
51 prion protein to PrP^{Sc} plays a key role in the neuropathological changes (44). Therefore,
52 compounds that inhibit PrP^{Sc} formation are considered as therapeutic candidates of the
53 diseases and many compounds have been reported to inhibit PrP^{Sc} formation in cell cultures
54 and cell-free systems [reviewed in (56)]. However, only a few of these inhibitors, such as
55 amphotericin B and its derivative (13), pentosan polysulfate (14), porphyrin derivatives (27)
56 certain amyloidophilic compounds (25) and FK506 (37) have been reported to prolong the
57 survival of prion-infected mice even when administered in the middle-late stage of infection,
58 but still before clinical onset. We recently reported that intraventricular infusion of anti-PrP
59 antibodies (50) slowed down the progression of the disease even when initiated just after
60 clinical onset. However, in addition to inhibition of PrP^{Sc} formation, protection of neurons
61 or restoration of degenerated neurons is thought to be important for functional recovery.

62 Bone marrow-derived mesenchymal stem cells (MSCs) differentiate into cells of
63 mesodermal origin such as adipocytes, osteoblasts and endothelial and muscle cells (41, 43).
64 In addition, MSCs are known to trans-differentiate into neuronal and glial cells. MSCs have
65 been shown to migrate to damaged neuronal tissues and to alleviate the deficits in
66 experimental animal models of cerebral ischemia (10), spinal cord injury (20), Parkinson's
67 diseases (19, 33) and amyotrophic lateral sclerosis (59). MSCs also secrete various

68 neurotrophic factors that may protect neuronal tissues from degradations as well as stimulate
69 the activity of endogenous neural stem cells (38). Therefore, despite their mesodermal
70 origin, MSCs are considered to be a candidate for cell-mediated therapy for
71 neurodegenerative diseases. One of the characteristics of MSCs is their migration to brain
72 lesions of neurodegenerative diseases including prion diseases (10, 19, 39, 51). This feature
73 may be of further use for cell-mediated therapy of neurodegenerative diseases, particularly for
74 prion diseases, Multiple sclerosis and Alzheimer's disease, which have diffuse pathological
75 lesions.

76 Since many cytokines, chemokines and adhesion molecules are involved in the homing of
77 immune cells (9, 36, 53), evidence that a variety of chemokines and growth factors, as well as
78 their cognate receptors have a pivotal role in the migration of MSCs has been accumulated.
79 These factors include CXCL12 and its receptor CXCR4 [(30, 40) and reviewed in (52)],
80 CCL2 (15, 62, 66), CCL3 (62), IL-8 (48, 62), hepatocyte growth factor (16), platelet-derived
81 growth factor-AB (PDGF-AB), insulin-like growth factor-1 (IGF-1), CCL5 and CCL22 (42),
82 and integrin β 1 (23). Regarding migration of MSCs to injury in the CNS, involvement of
83 CCL2 (61), CXCL12/CXCR4 and CX3CL1/CX3CR1 (24) have been reported. However,
84 knowledge of the mechanism by which MSCs migrate to pathological lesions of
85 neurodegenerative diseases is insufficient and further efforts are required to elucidate this
86 mechanism.

87 We recently reported that human MSCs (hMSCs) migrate to CNS lesions and prolong the
88 survival of mice infected with prion (51). In the present study we investigated factors that
89 are involved in the migration of hMSCs to brain lesions of prion diseases.

90

91

92 **MATERIALS AND METHODS**

93

94 **Cell culture**

95 Human bone marrow-derived MSCs that were immortalized with the human telomerase
96 catalytic subunit gene (26), and that stably expressed the LacZ gene [hMSCs, (51)] were used.

97 The hMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical
98 Co., St. Louis, MO) containing 10% fetal bovine serum (FBS) in a humidified atmosphere
99 under normoxic (21% O₂ and 5% CO₂) or hypoxic (2% O₂ and 5% CO₂) conditions at 37 °C.

100

101 **Mice and prion inoculation.**

102 All animal experiments were carried out according to protocols approved by the
103 Institutional Committee for Animal Experiments. Four-week-old female ICR mice were
104 purchased from CLEA Japan Inc. (Japan), and the mice were acclimatized for a week prior to
105 use. Brain homogenates [10% (w/w) in phosphate-buffered saline (PBS)] that were used for
106 inoculation were prepared from brains of mice infected with the Chandler prion strain at the
107 terminal stage of the disease and from age-matched uninfected mice. Mice were
108 intracerebrally inoculated with 20 µl of 10% brain homogenates.

109

110 ***In vitro* migration assay**

111 To examine the migration of hMSCs *in vitro*, the brains of infected mice 120 days post
112 inoculation (dpi) with the Chandler strain, or of age-matched mock-infected mice, were
113 homogenized to 20% (w/w) in DMEM. The homogenates were centrifuged at 10,000 × g for

114 10 min at 4 °C, and the resulting supernatants were passed through a 0.22- μ m pore size filter.
115 Aliquots of the brain extracts were stored at -80 °C until use. Migration of hMSCs to the
116 brain extracts was assessed using a QCMTM chemotaxis cell migration assay kit (Chemicon,
117 Temecula, CA). The hMSCs that were starved for a day in serum-free medium were
118 harvested, and 300 μ l of cell suspension (5×10^4 cells) was added to the insert well. The
119 lower chambers were supplied with serum-free DMEM containing 1% brain extract.
120 Twenty-four hours after incubation, hMSCs on the poly-carbonate membrane (pore size 8.0
121 μ m) were stained with the cell stain solution provided in the kit. Non-migratory cells that
122 stayed on the upper side of the poly-carbonate membrane were removed using a cotton swab.
123 The migrated hMSCs, which had passed through the pores and clung to the under-side of the
124 membrane, were counted using the NIH Image J program. Three random
125 high-magnification (100 \times) light microscopic images were captured using the Olympus BX-51
126 microscope, and were used for cell counting.

127 To examine the involvement of receptors expressed on hMSCs in their migration, hMSCs
128 were pre-incubated with antibodies against specific receptors for 30 min before adding the
129 insert wells. To examine the involvement of chemokines and growth factors in migration,
130 1% brain homogenates were incubated with antibodies against specific chemokines and
131 growth factors for 30 min at 4 °C prior to adding to the insert wells. Antibodies against the
132 following proteins were purchased from the indicated companies: PDGF- $\alpha\beta$ receptor
133 (PDGF- $\alpha\beta$ R, ab34074); CCR2 (ab1668); CCR4 (ab1669); CX3CR1 (ab7201); CXCR3 (clone
134 2Ar1) and CCL3 (ab10381), all from Abcam (Cambridge, MA); IGF-1 receptor (IGF-1R;
135 clone 33255); CXCR4 (clone 12G5); IGF-1 (AF791); CCL2 (clone 123602); CCL4
136 (AF-451-NA); CCL5 (AF-478); CCL7 (AF-456-NA); CCL17 (AF-529); CCL24 (clone

137 106521); CX3CL1 (clone 126315); CXCL10 (AF-466-NA); CXCL12 (clone 79014) and
138 CXCL13 (AF-470), all from R&D systems (Minneapolis, MN); CCR1 (clone #141-2) and
139 CCR3 (clone 444-11), both from MRL; CCR5 (Sigma Chemical Co., clone 45523.111);
140 PDGF-AB (Millipore, Billerica MA, #06-127). All antibodies were used at a concentration
141 of 10 µg/ml. Migration assays were performed as three independent experiments (each
142 experiment was carried out in triplicate).

143

144 **Transplantation of hMSCs**

145 The hMSCs were transplanted into the thalamus as described previously (51). Briefly,
146 after anesthesia, mouse scalps were incised. The mice were then placed onto a stereotaxic
147 apparatus (Narishige, Japan), and burr holes were drilled to accommodate stereotaxic
148 placement into the thalamus (Bregma - caudal 2.0 mm, lateral 2.1 mm and depth 3.2 mm).
149 The hMSCs (1×10^5 cells in 2 µl DMEM) were transplanted over a period of 15 min using a
150 Hamilton syringe with a 31-gauge needle.

151

152 **Flow cytometric analysis**

153 The hMSCs were treated with 1mM EDTA and dispersed in 0.5% FBS in PBS (FBS-PBS)
154 by pipetting. The cells were then incubated with primary mouse antibodies against IGF-1R,
155 CCR1, CCR3, CCR5, CXCR3 and CXCR4; primary goat antibodies against PDGF-αβR,
156 CCR2 and CCR4, and with a primary rabbit antibody against CX3CR1 (all 1:200 dilution) in
157 0.5% FBS-PBS for 30 min on ice. The primary antibodies were omitted for negative
158 controls. The cells were washed three times with 0.5% FBS-PBS and incubated with
159 anti-mouse Alexa Fluor 546, anti-goat Alexa Fluor 555 or anti-rabbit Alexa Fluor 555

160 (Molecular Probes, Eugene, OR) (1:1000 dilution) for 30 min on ice. After washing, the
161 cells were stained with 5 µg/ml of propidium iodide (Molecular Probes) in PBS for 5 min, and
162 analyzed using an EPICS XL-ADC flow cytometer (Beckman Coulter, Miami, FL).

163

164 **Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

165 Total RNA was obtained from hMSCs or from mouse brains using Trizol Reagent
166 (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized from 2.5
167 µg of the total RNA using a First-strand synthesis kit (Amersham Biosciences, UK) according
168 to the manufacturer's instructions. Quantitative PCR was carried out using a TaqMan assay.
169 The amplification reaction mixtures contained template cDNA, 1× pre-designed TaqMan
170 Gene Expression Assays and 1× TaqMan Universal PCR Master Mix (Applied Biosystems,
171 Foster City, CA) in a final reaction volume of 20 µl. The following TaqMan gene expression
172 assays were purchased from Applied Biosystems; human genes for PDGF-αβR (assay ID;
173 Hs00182163), CCR3 (Hs00266213), CCR4 (Hs99999919), CCR5 (Hs99999149), CX3CR1
174 (Hs00365842), CXCR3 (Hs01847760) and CXCR4 (Hs00607978), and mouse genes for
175 CCL3 (Mm00441259), CCL4 (Mm00443111), CCL5 (Mm01302427), CCL7 (Mm00443113),
176 CCL17 (Mm01244826), CX3CL1 (Mm00436454), CXCL10 (Mm00445235) and CXCL12
177 (Mm00445553). The human RNase P gene or the mouse glyceraldehyde-3-phosphate
178 dehydrogenase (GAPDH) gene was amplified as an internal marker using human RNase P
179 control reagents (Applied Biosystems, 4316844) or TaqMan rodent GAPDH control reagents
180 (Applied Biosystems, 4308313). TaqMan assays were carried out using an ABI PRISM
181 7900HT Sequence Detection System (Applied Biosystems). The amplification profiles were
182 analyzed using the threshold cycle (CT) relative quantification method, and were normalized

183 to the expression of the human RNase P control gene or the mouse GAPDH gene, which were
184 used as human and mouse reference genes as described previously (58).

185

186 **Immunofluorescence assay (IFA)**

187 IFA was performed on hMSCs cultured in an 8-well chamber slide (Nalge Nunc,
188 Naperville, IL) or on cryosections (5 μ m thick) of mouse brains after transplantation of
189 hMSCs. The cells or sections were fixed with cold methanol for 20 min at -20 °C, and were
190 treated with PBS containing 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween-20)
191 (PBST) for 10 min. After blocking with 5% FBS in PBST for 30 min, the cells or sections
192 were incubated for 1 h with primary antibodies for the receptors described above using a
193 1:500 dilution. After washing with PBST, they were then incubated with a 1:2000 dilution
194 of anti-mouse Alexa Fluor 546, anti-goat Alexa Fluor 555 or anti-rabbit Alexa Fluor 555 for 1
195 h at room temperature. To investigate the co-localization of hMSCs with their receptors, the
196 sections were incubated for 90 min with a mouse anti- β -gal antibody (Cat No. Z3783,
197 Promega, Madison, WI) conjugated with Alexa Fluor 488 (51). After washing with PBST,
198 samples were then mounted with Vectashield containing 4'-6-diamidino-2-phenylindole
199 (DAPI; Vector Laboratories, Burlingame, CA). Samples were observed under a Nikon C1
200 laser confocal microscope.

201

202

203 **RESULTS**

204

205 **Migration of hMSCs to the brain extracts**

206 Hypoxic pre-conditioning of MSCs is reported to enhance their migratory activity (2, 22).
207 To determine the effects of oxygen conditions on the migration of hMSCs to brain
208 homogenates of mock- or prion-infected mice, hMSCs that were cultured under normoxic or
209 hypoxic conditions were analyzed using an *in vitro* migration assay. The hMSCs that were
210 pre-cultured under hypoxic conditions migrated to brain extracts of prion-infected mice more
211 efficiently than those pre-cultured under normoxic conditions (Fig. 1A). Quantitative
212 analysis revealed that twice as many hMSCs migrated to the brain extracts of prion-infected
213 mice than to those of mock-infected mice following normoxic pre-conditioning, while 2.7
214 times more hMSCs migrated to the brain extracts of prion-infected mice following hypoxic
215 pre-conditioning (Fig. 1B). No differences were observed in the migration of hMSCs to the
216 brain extracts of mock-infected mice between normoxic and hypoxic pre-conditioning. We
217 therefore used hMSCs that were pre-cultured under hypoxic conditions for all subsequent
218 experiments to facilitate discrimination between subtle differences in cell migration. These
219 hMSCs showed a time-dependent increase in migration for up to 24 h (Fig. 1C), and their
220 migration increased in a brain-extract concentration-dependent manner (Fig. 1D). Migration
221 of hMSCs to 0.01% and 0.001% brain homogenates of prion-infected mice was observed,
222 whereas only migration to 1% brain homogenates, but not to lower percentages of brain
223 extracts of mock-infected mice was observed.

224

225 **Chemotactic factors involved in the migration of hMSCs**

226 The chemokine CXCL12 and its receptor, CXCR4, are known to be involved in the
227 chemotactic migration of MSCs to brain lesions of neurodegenerative diseases (24, 63).
228 However, as MSCs express a variety of receptors for growth factors, chemokines and

229 cytokines that are associated with cell migration (8, 52), it was anticipated that other
230 cytokines, chemokines and growth factors also play a role in the migration of MSCs. We
231 therefore selected 10 receptors for chemokines and growth factors, and 13 of their ligands, as
232 indicated in Table 1, and analyzed their involvement in hMSC migration using an *in vitro*
233 migration assay (Fig. 2). These factors were chosen with reference to cell surface expression
234 of those receptors on MSCs in previous studies (50), and to the pathway and networks of
235 receptors and their ligands obtained using Ingenuity Pathway Analysis 5.0 (Ingenuity System
236 Inc., Redwood City, CA). The migration of hMSCs to the brain extracts of prion-infected
237 mice was significantly decreased when hMSCs were pre-treated with antibodies against
238 CCR3, CCR4, CCR5, CXCR3 and CXCR4, compared with the migration of non-treated
239 hMSCs ($p < 0.05$, Dunnett's tests) (Fig. 2A). When antibodies against the ligands of these
240 receptors were tested (Table 1), the migration of hMSCs was significantly decreased by
241 treatment of the brain extracts with antibodies against the ligands for CCR3 (CCL5, CCL7
242 and CCL24), CCR5 (CCL3, CCL4 and CCL5), CXCR3 (CXCL10) and CXCR4 (CXCL12)
243 ($p < 0.05$) (Fig. 2B). Neither the antibody against CX3CR1, nor that against its
244 corresponding ligand, CX3CL1, decreased the migration of hMSCs. In addition, the
245 antibody against CCR4 decreased hMSC migration, whereas the antibody against its ligand,
246 CCL17, did not. When the involvement of growth factors and their receptors in migration
247 was analyzed, antibodies against the PDGF- α βR or the IGF-1R did not decrease migration,
248 whereas antibodies against their respective ligands, PDGF-AB and IGF-1, did decrease
249 migration. In addition, an antibody against CCR2 or its ligand CCL2 did not decrease
250 migration, but an antibody against CCL7, another ligand for CCR2, did decrease migration.
251 Taking into account of biological relationship between the receptors and ligands that were

252 analyzed here, the migration assays suggested that at least CCR3, CCR5, CXCR3 and
253 CXCR4, and their ligands were involved in the migration of hMSCs to the brain extracts of
254 prion-infected mice.

255

256 **The expression of chemokine and chemokine receptor genes.**

257 We next examined the expression of chemokine genes in the brains of prion-infected mice
258 and that of chemokine receptor genes in hMSCs treated with the brain extracts of
259 prion-infected mice. For the latter analysis, hMSCs were incubated with DMEM containing
260 1% brain extracts of prion- or mock-infected mice. Twenty-four hours after incubation, total
261 RNA was recovered and gene expression was analyzed using qRT-PCR. The expression of
262 CCR3, CCR4, CCR5 and CXCR4 genes was elevated 2- to 9-fold in hMSCs treated with the
263 brain extracts of prion-infected mice compared to their expressions in hMSCs that were
264 treated with the brain extracts of mock-infected mice (Fig. 3A). In contrast, the expression
265 of PDGF- $\alpha\beta$ R, CX3CR1 and CXCR3 genes was not up-regulated by stimulation of the
266 hMSCs with brain extracts from prion-infected mice.

267 The expression of chemokine genes in the brains of prion-infected mice or of age-matched
268 control mice was also analyzed at 120 dpi. The expression of CCL3, CCL4, CCL5 and
269 CXCL10 genes was up-regulated by 10-fold (CXCL10, CCL5) to nearly 50-fold (CCL3,
270 CCL4) in the brains of prion-infected mice (Fig. 3B). In addition, the mRNA levels of
271 CCL7 and CCL17 (a ligand for CCR4) were moderately increased (about 3 to 6-fold) in the
272 brains of prion-infected mice. In contrast, expression of the CX3CL1 and CXCL12 genes
273 was not up-regulated.

274

275 **Expression of chemokine receptors on hMSCs.**

276 Flowcytometric analysis and IFA were carried out to confirm the expression of chemokine
277 and growth factor receptors on hMSCs. The hMSCs treated with brain extracts of
278 mock-infected mice expressed PDGF- $\alpha\beta$ R, CCR3, CCR4, CXCR4 and CXCR3 on the cell
279 surface. Of these receptors, expression of CCR3, CCR4 and CXCR4 was increased by
280 stimulation with brain extracts of prion-infected mice, whereas no increase in expression of
281 PDGF- $\alpha\beta$ R and CXCR3 was observed. Although it is difficult to distinguish signals on the
282 plasma membrane from those in the cytoplasm using IFA, a similar tendency was observed in
283 IFA; CCR3, CCR4 and CXCR4 fluorescent signals appeared to be more intense in cells
284 treated with brain extracts of prion-infected mice than in cells treated with brain extracts of
285 mock-infected mice. In contrast to the expression of these receptors, hMSCs expressed a
286 trace level of CX3CR1, and CCR5 was not detectable on the cell surface. However, the
287 expression of CCR5 was specifically induced in response to brain extracts of prion-infected
288 mice (Fig. 4A). The prion-specific induction of CCR5 expression was also confirmed by
289 IFA (Fig. 4B).

290

291 **Differential expression of chemokine receptors on hMSCs after transplantation into the**
292 **brains of prion-infected mice**

293 It is known that MSCs transplanted into the left hippocampus or thalamus migrate to the
294 contralateral (right) hippocampus through the corpus callosum (3, 19, 51). Therefore, we
295 hypothesized that receptors expressed on hMSCs in the corpus callosum are possibly involved
296 in the migration of hMSCs to neuropathological lesions. We therefore transplanted hMSCs
297 into the left thalamus of prion- or mock-infected mice at 120 dpi, and analyzed the expression

298 of growth factor/chemokine receptors on hMSCs. Two days after transplantation of hMSCs
299 into the left thalamus of prion-infected mice, hMSCs in the transplanted region expressed
300 CCR1, CCR3, CCR4, CCR5, CX3CR1, CXCR3 and CXCR4 (Fig. 5A) as well as PDGF- $\alpha\beta$ R,
301 IGF-1R and CCR2 (data not shown). However, the hMSCs that were transplanted into the
302 mock-infected mice showed weak expression of these receptors except for CXCR3,
303 suggesting that the expression of these chemokine receptors was increased by stimulation
304 with factors produced in the brains of prion-infected mice. At 2 days post-transplantation,
305 hMSCs in the corpus callosum of prion-infected mice still strongly expressed CCR3, CCR5,
306 CXCR3 and CXCR4, but expression of CCR1, CX3CR1 and CCR4 appeared to be lower than
307 that on hMSCs in the transplanted area (Fig. 5B). The expression of chemokine receptors on
308 hMSCs in the contralateral hippocampus also differed from that on hMSCs in the transplanted
309 region and in the corpus callosum; hMSCs preferentially expressed only CCR1, CX3CR1 and
310 CXCR4 in the contralateral hippocampus at one week post-transplantation (Fig. 5B). These
311 data suggest that CCR1, CX3CR1 and CXCR4 may be associated with specific activities of
312 hMSCs after their migration to the target lesions.

313

314

315 **DISCUSSION**

316

317 MSCs are known to migrate to neuropathological lesions of neurodegenerative diseases (8,
318 54). The migrated MSCs can contribute to the functional recovery of damaged nervous
319 tissues by secretion of various trophic factors (12), neuronal differentiation or cell fusion (1,
320 29), and stimulation of the proliferation and differentiation of endogenous neural stem cells

321 (38). To date, transplantation of MSCs has been reported to ameliorate the symptoms not
322 only of the experimental animal models of neurodegenerative diseases (10, 19, 20, 33, 59),
323 but also of human patients with multiple system atrophy (32), amyotrophic lateral sclerosis
324 (35) or stroke (4). The involvement of CXCL12 and its cognate receptor CXCR4 in MSC
325 migration to injured tissues is well-established (17) in spite of some exceptions (23). *In vitro*
326 migration assays reported to date have identified growth factors and chemokines that are
327 possibly involved in MSC migration (42, 49, 60, 61). However, the mechanisms of MSCs
328 migration are expected to differ with tissue microenvironments induced by diseases.
329 Targeting of MSCs to neuropathological lesions is essential for functional recovery, therefore,
330 an understanding of the mechanisms that underlie the migration of MSCs to lesions in the
331 CNS could contribute to the development of MSC-mediated cell therapy by facilitating
332 site-specific migration of MSCs. The involvement of CCL2, CXCL12/CXCR4 and
333 CXCL1/CX3CR1 in the migration of MSCs to brain lesions has been reported (24, 62, 63).
334 However, the mechanisms that underlie migration of MSCs to neuropathological lesions are
335 largely unknown.

336 We recently showed that hMSCs can migrate to neuropathological lesions induced by prion
337 propagation (51). Since brain extracts of prion-infected mice were considered to contain
338 chemoattractive factors (5, 46, 57, 65), we analyzed factors that induce hMSC migration by
339 blocking experiments using antibodies against receptors for growth factors and chemokines
340 and their ligands. To increase the accuracy of the interpretation of *in vitro* migration assays,
341 we defined a ligand/receptor interaction as chemoattractive if antibodies against both the
342 ligand and its cognate receptor reduced the migration of hMSCs. Based on this criterion, we
343 expected that CCR3, CCR5, CXCR3 and CXCR4, and their ligands are possibly involved in

344 the migration of hMSCs to the brain extracts of prion-infected mice (Fig. 2). The
345 involvement of CXCL12/CXCR4 signaling in hMSCs migration is consistent with findings in
346 hypoglossal nerve injury (24), ischemic (63) and glioma (11) models. Although an effect of
347 CCR3, CCR5 or CXCR3 on MSC migration in brain injury has not been reported, CXCR3
348 and CCR5 are known to modulate resident microglial migration to brain lesions (6, 45). In
349 prion diseases, impairment of microglial migration, associated with the increased
350 accumulation of PrP^{Sc} but prolongation of survival, has been reported in CXCR3 gene
351 deficient mice infected with prions (47). Microglial recruitment in retina after intraocular
352 injection of homogenates from prion-infected neuroblastoma cells was inhibited by CCR5
353 antagonist, suggesting the involvement of CCR5 in microglial response to prion infection (34),
354 although ablation of CCR5 gene did not influence the incubation period after prion infection
355 (55). Since MSCs are able to migrate to brain lesions, the mechanisms by which they do so
356 are expected to show some similarity with the mechanisms that underlie microglial migration.
357 CCL2 has been reported to mediate MSC migration to ischemic brain lesions (62). However,
358 neither an anti-CCL2 antibody, nor an antibody against its receptor, CCR2, reduced hMSC
359 migration to brain extracts of prion-infected mice, implying that CCL2/CCR2 interaction may
360 not mediate the migration of MSCs to prion-specific brain lesions. PDGF-AB and IGF-1
361 have been reported to be strong chemoattractants for MSC migration *in vitro* (42). In the
362 present study, antibodies against these two growth factors reduced hMSC migration, but
363 antibodies against their receptors did not inhibit hMSC migration despite the surface
364 expression of these receptors on hMSCs (Fig. 4). Because of the complexity of the brain
365 extracts and the limitations of blocking experiments, the lack of inhibition by these antibodies
366 against those receptors does not necessarily mean that these ligands/receptor interactions have

367 no functional relevance. Further detailed analysis will define additional factors that possibly
368 mediate the migration of MSCs in response to damage of nervous tissue caused by prion
369 propagation.

370 Regarding the expression of chemokine genes, the gene expression of CCR5 ligands
371 (CCL3, CCL4 and CCL5), CCR3 ligands (CCL5 and CCL7) and the ligand for CXCR3
372 (CXCL10) was up-regulated. The up-regulation of CXCL10 gene in the CNS following
373 viral infection has been reported to attract T lymphocytes bearing CXCR3 into the CNS (64),
374 suggesting that MSCs share some mechanism of trafficking with lymphocyte trafficking.
375 The hMSCs transplanted into the brains of mock-infected mice do not migrate, and the degree
376 of hMSC migration in the brains of prion-infected mice correlates with the progression of
377 neuropathological lesions (51). These results indicate that constitutive expression levels of
378 chemokines and/or growth factors in the brain do not, but increased levels of those factors do,
379 induce hMSC migration. Therefore, although CXCR4 and its ligand CXCL12 have been
380 reported to play an important role in migration of MSCs in the CNS (24, 63), the lack of
381 up-regulation of CXCL12 gene expression in prion-infected mice suggests that
382 CXCL12/CXCR4 interaction does not have an important role in the initial event on attraction
383 of hMSCs. Indeed, cell migration is dependent on a multitude of signals. Therefore,
384 cytokines, chemokines and growth factors, whose expression is up-regulated in the brains of
385 prion-infected mice, e.g., the CXCL10, CCL3-5 and CCL7 that we analyzed in this study, as
386 well as other factors reported previously (5, 57, 65), will stimulate hMSCs to initiate
387 migration.

388 The corpus callosum is known to be one of the sites through which MSCs transplanted into
389 one hemisphere migrate to the contralateral hemisphere (3, 19). We recently reported that

390 hMSCs transplanted into the left thalamus of prion-infected mice were detected in the corpus
391 callosum, the contralateral hippocampus and thalamus 2 days after transplantation (51). This
392 result suggests that the hMSCs that were detected in the corpus callosum were migrating to
393 the brain lesions in the contralateral hemisphere. Based on this idea, we therefore analyzed
394 the expression of chemokine receptors on hMSCs that were transplanted into prion-infected
395 mice. Interestingly, in agreement with the interpretation of the *in vitro* migration assays,
396 hMSCs in the corpus callosum clearly expressed CCR3, CCR5, CXCR3 and CXCR4 2 days
397 after transplantation (Fig. 5B), suggesting the involvement of these chemokine receptors in
398 the migration of hMSCs *in vivo*. In contrast to hMSCs in the corpus callosum, hMSCs that
399 had migrated to the contralateral hippocampus showed reduced expression of CCR3, CCR5
400 and CXCR3, whereas strong expression of CXCR4 was still observed. These results suggest
401 that CXCR4 plays a role not only in migration, but also in regulating hMSC activity following
402 chemotactic migration. A role in the regulation of hMSC activity following chemotactic
403 migration may also apply to the expression of CCR1 and CX3CR1; the weak expression of
404 CCR1 and CX3CR1 on hMSCs in the corpus callosum (Fig. 5B) appears to be consistent with
405 the results of the *in vitro* migration assay (Fig. 2). However, in contrast, these receptors
406 were clearly expressed on hMSCs in the transplanted thalamus at 2 days post-transplantation
407 and in the contralateral hippocampus of prion-infected mice a week after transplantation,
408 implying that CCR1 and CX3CR1 may play a role in regulating MSC activity after migration
409 to the targeted site.

410 Although neuroprotective functions of MSCs that have migrated to brain lesions are not
411 fully understood, the temporal and spatial differences in the expression of chemokine
412 receptors on hMSCs transplanted into the brains of prion-infected mice that we observed in

413 this study is intriguing, *i.e.*, hMSCs that are considered to be in the process of migration in the
414 corpus callosum expressed CCR3, CCR5 and CXCR3, whereas hMSCs that had migrated to
415 the target site showed reduced expression of these receptors but elevated expression of CCR1
416 and CX3CR1. The stimulation of CX3CR1 by its ligand CX3CL1 plays a role in
417 modulating the release of pro-inflammatory cytokines from microglia (7, 21) and in
418 producing neuroprotective substances (31). Therefore, expression of CX3CR1 on MSCs
419 that had migrated to the contralateral hippocampus might be an indicator of functional
420 alteration of hMSCs, *i.e.*, alteration from active migration towards the target site to exhibition
421 of neuroprotective potential. The hMSCs that had migrated to the contralateral hippocampus
422 still expressed CCR1, CX3CR1 and CXCR4 at 3 weeks post-transplantation (data not shown).
423 We have previously shown that hMSCs transplanted into prion-infected mice efficiently
424 produced various neurotrophic factors at 1 to 3 weeks post-transplantation (51). It is thus
425 conceivable that signaling via these receptors may facilitate alteration of the phenotype of
426 hMSCs to that of a neuroprotective phenotype following chemotactic migration. The
427 interaction between endogenous nucleotide and purinergic receptors on microglia is known to
428 regulate the activation state of microglia. Interaction of ATP with the P2Y₁₂ receptor induces
429 microglial chemotaxis to local CNS injury. However, a decrease in expression of the P2Y₁₂
430 receptor accompanies morphological change of microglia from a ramified to an amoeboid
431 state (18). In contrast, up-regulation of the expression of P2Y₆ receptors by neuronal
432 damage and interaction of UDP released from damaged neurons with the P2Y₆ receptor
433 triggers phagocytic activity of microglia (28). It is therefore of interest to investigate
434 whether a temporal and spatial change in the expression profile of chemokine receptors,
435 similar to the control of microglial activity by endogenous nucleotides and purinergic

436 receptors, accompanies alteration in the activation states of MSCs.

437 In this study, we showed that, in addition to CXCR4, the chemokine receptors CCR3,
438 CCR5 and CXCR3 are involved in the migration of hMSCs to brain lesions caused by prion
439 infection. The fact that the results of the *in vitro* migration assay are consistent with the
440 expression of chemokine receptors on hMSCs transplanted into the brains of prion-infected
441 mice provides strong evidence for the involvement of these chemokine receptors in the
442 migration of hMSCs. Although factors that regulate the migration of hMSCs in the CNS
443 may vary with diseases and with the microenvironment of the lesions, our results provide an
444 insight into the mechanism of MSC migration towards neuropathological lesions. In
445 addition, our results also show that comparison of MSC receptor expression in migrating
446 MSCs with that of MSCs that had homed to the targeted site provides a clue to identification
447 of factors that facilitate the homing of MSCs. Further investigation of the host cells that
448 produce relevant ligands for those receptors in response to the progression of
449 neuropathological lesions, the temporal order of receptor expression on MSCs, and of the
450 mechanisms that regulate the activity of MSCs, may facilitate the application of MSCs to
451 prion diseases.

452

453

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682

683

684 **FIGURE LEGENDS**

685

686 **Figure 1.** Chemotactic migration of hMSCs to brain extracts of mock- or prion-infected
687 mice. The migration of hMSCs to brain extracts of mock- or prion-infected mice that were
688 prepared at 120 dpi was analyzed using a QCMTM 96-well cell migration assay kit. (A)
689 hMSCs migrated to the underside of the membrane of the insert well. The hMSCs that were
690 pre-cultured under normoxic or hypoxic conditions (Normoxic or Hypoxic) were added to the
691 insert well, and the lower chambers were supplied with serum-free DMEM containing 1%
692 brain extracts of mock- or prion-infected mice (Mock or Prion). Twenty-four hours after
693 incubation, hMSCs that had passed through the pores of the membrane and clung to its

694 underside were stained (upper panel). Stained cells with a size $> 200 \mu\text{m}^2$ were selected
695 (lower panel) and were counted using the Image NIH J program. Bar = $100 \mu\text{m}$. (B)
696 Quantification of migrated hMSCs. The migration of hMSCs, assayed as described in (A)
697 was quantified. Three random areas ($3.84 \times 10^5 \mu\text{m}^2/\text{area}$) of the underside of the membrane
698 were photographed and the number of hMSCs was counted. The graphs show cell counts
699 per $3.84 \times 10^5 \mu\text{m}^2$ (mean and SD, $n = 3$). Black and white bars indicate the number of
700 hMSC that migrated to 1% brain extracts of mock- and prion-infected mice, respectively.
701 (C) Time-dependent increase in hMSC migration. The migration assay was performed for 4,
702 15 and 24 h. (D) Dose-dependency of hMSC migration. The migration of hMSCs to
703 various doses (0.001, 0.01, 0.1 and 1%) of the brain extracts was analyzed. The graph shows
704 cell counts per $3.84 \times 10^5 \mu\text{m}^2$ at the underside of the membrane (mean and SD, $n = 3$).

705

706 **Figure 2.** Screening of chemotactic factors involved in the migration of hMSCs. The
707 migration of hMSCs to 1% brain extracts of prion-infected mice was assessed after
708 pre-treatment of hMSCs with antibodies against receptors for chemokines or growth factors
709 (A), or after pre-treatment of brain extracts with antibodies against growth factors or
710 chemokines (B). Migration assays, in which antibody pre-treatments of hMSCs and brain
711 extracts were omitted, were assigned as control (Cont). The graphs show relative numbers
712 of hMSCs that migrated to brain extracts of prion-infected mice compared to numbers of
713 hMSCs that migrated to brain extracts in control experiments. Means and SD from three
714 independent assays (each assay was carried out in triplicate) are shown. Asterisks: $p < 0.05$
715 (Dunnett's *post hoc* test).

716

717 **Figure 3.** Expression of chemokine receptor genes in hMSCs and chemokine genes in the
718 brains of prion-infected mice. (A) Expression of chemokine and growth factor receptor
719 genes in hMSCs stimulated with brain extracts of prion-infected mice. The hMSCs were
720 incubated for a day in DMEM containing 1% brain extracts of prion-infected mice prepared at
721 120 dpi or of age-matched mock-infected mice. Expression of the mRNA of the indicated
722 genes was then analyzed using qRT-PCR. The graph shows the fold increase in gene
723 expression in hMSCs incubated with brain extracts of prion-infected mice compared to
724 hMSCs incubated with brain extracts of mock-infected mice. The results of two independent
725 experiments are shown. (B) Expression of chemokine genes in the brains of prion-infected
726 mice. The graph shows fold increase in gene expression in the brains of prion-infected mice
727 at 120 dpi compared to the brains of age-matched mock-infected mice. Mean and SD (n = 3)
728 of the fold increase are shown.

729

730 **Figure 4.** Expression of chemokine receptors on hMSCs. (A) Flow cytometric analysis.
731 The expression of chemokine receptors on the cell surface was examined after incubation of
732 the cells with brain extracts of mock- (black) or prion- (red) infected mice prepared at 120 dpi.
733 The grey histograms indicate the negative control (omitted primary antibodies). (B) IFA.
734 The hMSCs that were incubated with brain extracts of mock- or prion-infected mice (Mock or
735 Prion) for 24 h in 8-chamber slides were stained with antibodies against chemokine receptors
736 (red), and were counterstained with DAPI (blue). Bar = 20 μ m.

737

738 **Figure 5.** Expression of chemokine receptors in hMSCs transplanted into the brains of
739 mock- or prion-infected mice. The hMSCs (1×10^5 cells) were transplanted into the left

740 thalamus of mock- or prion-infected mice at 120 dpi and cryosections of mouse brains were
741 prepared at 2 days or a week post-transplantation. Sections were double stained with
742 anti- β -gal antibodies conjugated with Alexa fluor 488 (green) for staining of hMSCs and with
743 antibodies against chemokine receptors (red). Nuclei were counterstained with DAPI (blue).
744 (A) Expression of chemokine receptors in hMSCs at the transplanted area. Images of the left
745 thalamus (transplanted side) of mock- and prion-infected mice were taken at 2 days
746 post-transplantation. (B) Expression of chemokine receptors in hMSCs in the corpus
747 callosum and the right hippocampus. Images of the corpus callosum and the right
748 hippocampus (contralateral side) of prion-infected mice were taken at 2 days and one week
749 post-transplantation, respectively. Bar = 20 μ m.

750 Table 1. Growth factor and chemokine receptors, and their corresponding ligands^a

Receptors	Corresponding ligands
HGFR ^b	HGF
IGF-1R ^c	IGF-1
PDGF- $\alpha\beta$ R ^d	PDGF-AB
CCR1	CCL3, CCL5, CCL7, CCL13
CCR2	CCL2, CCL7, CCL8, CCL13
CCR3	CCL5, CCL7, CCL8, CCL11, CCL24
CCR4	CCL17, CCL22
CCR5	CCL3, CCL4, CCL5, CCL8, CCL11
CX3CR1	CX3CL1
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13

751 ^a The table was referred from Spaeth *et al* (52).

752 ^b HGFR, hepatocyte growth factor receptor

753 ^c IGF-1R, insulin-like growth factor receptor

754 ^d PDGF- $\alpha\beta$ R, platelet-derived growth factor- $\alpha\beta$ receptor

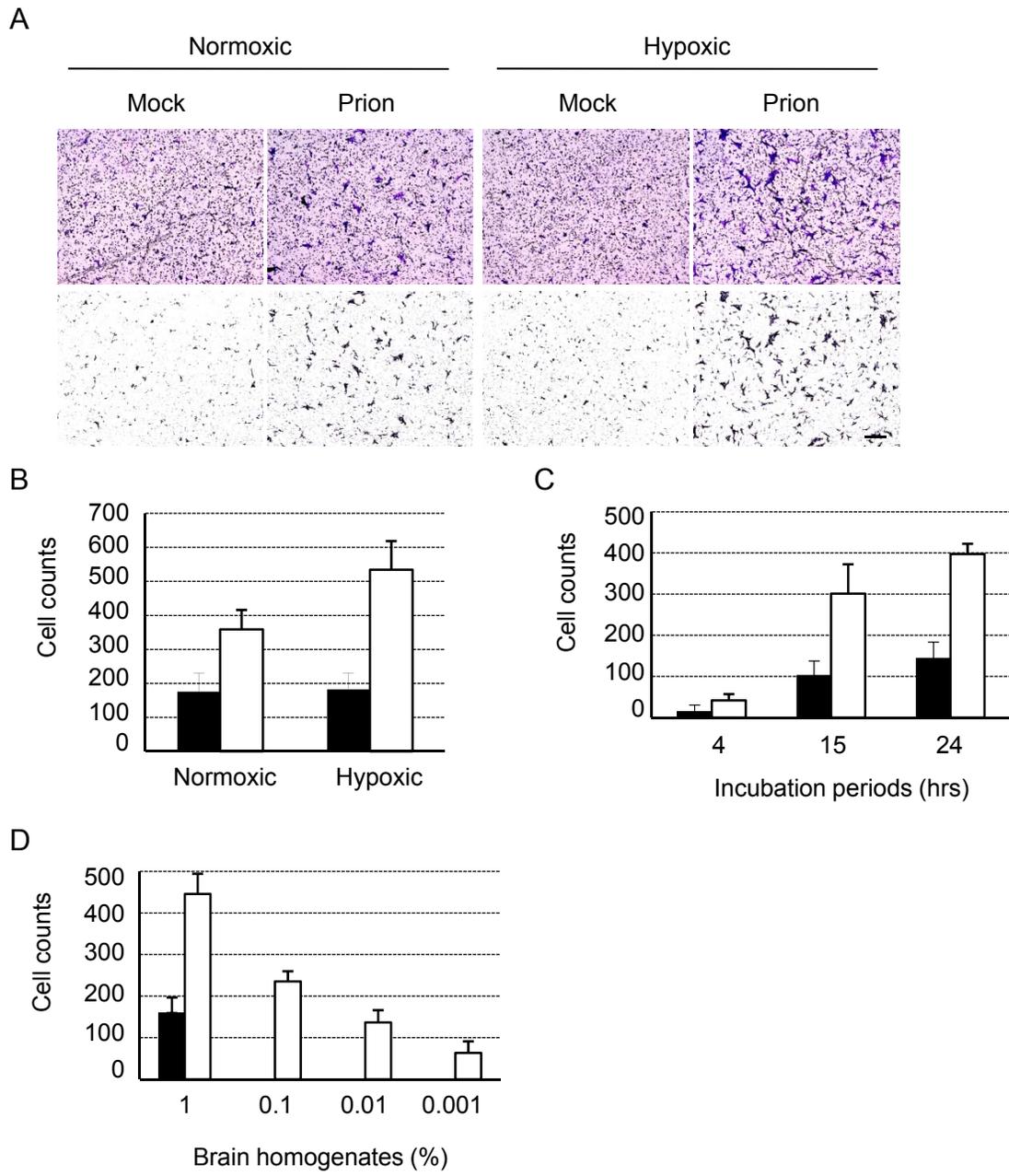
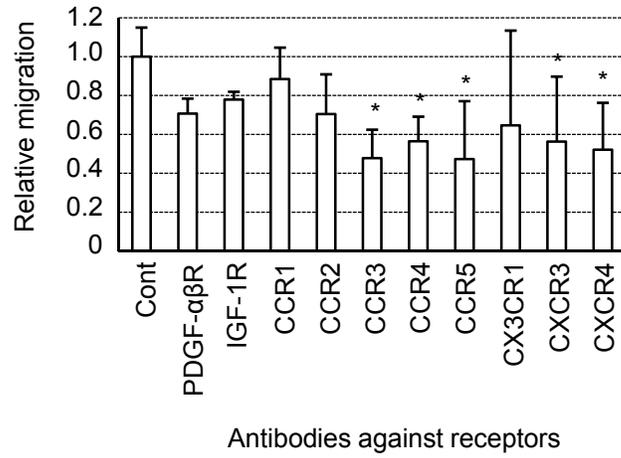
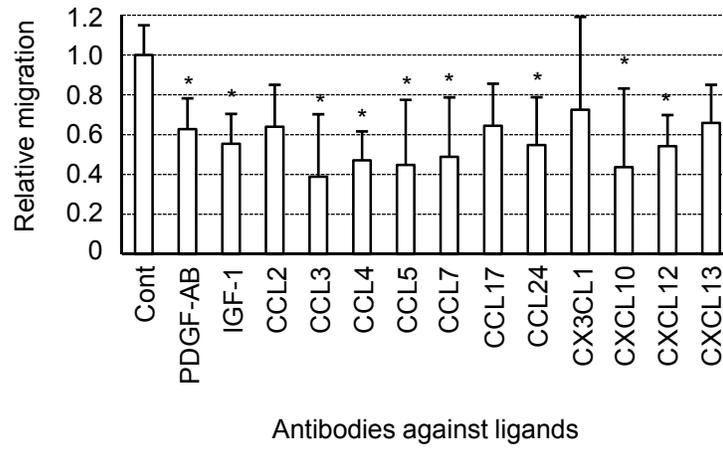


Fig. 1 Song *et al.*

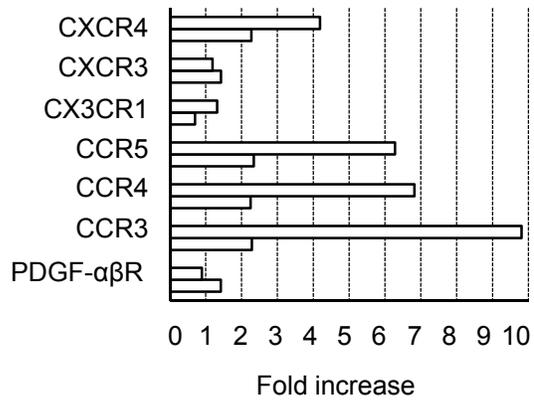
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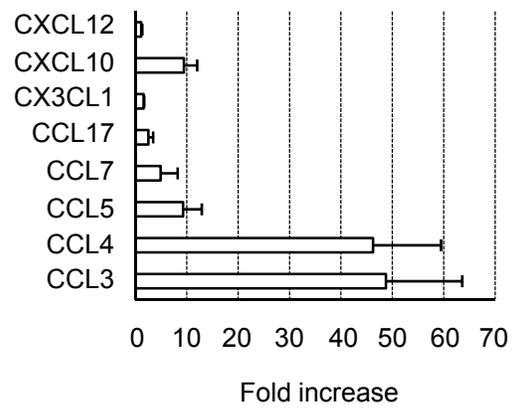
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B



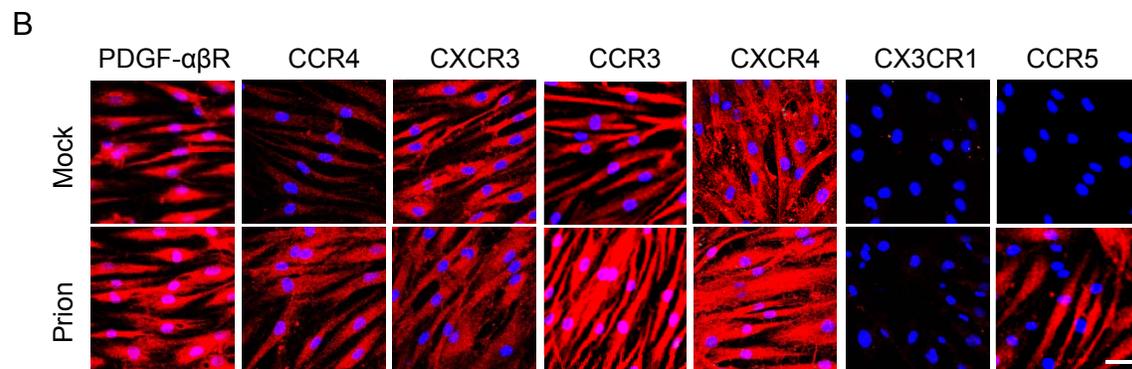
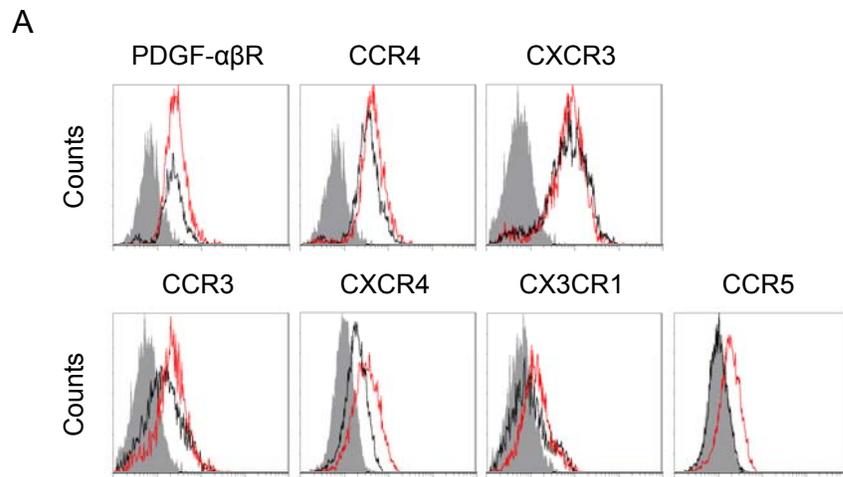


Fig. 4 Song *et al.*

A

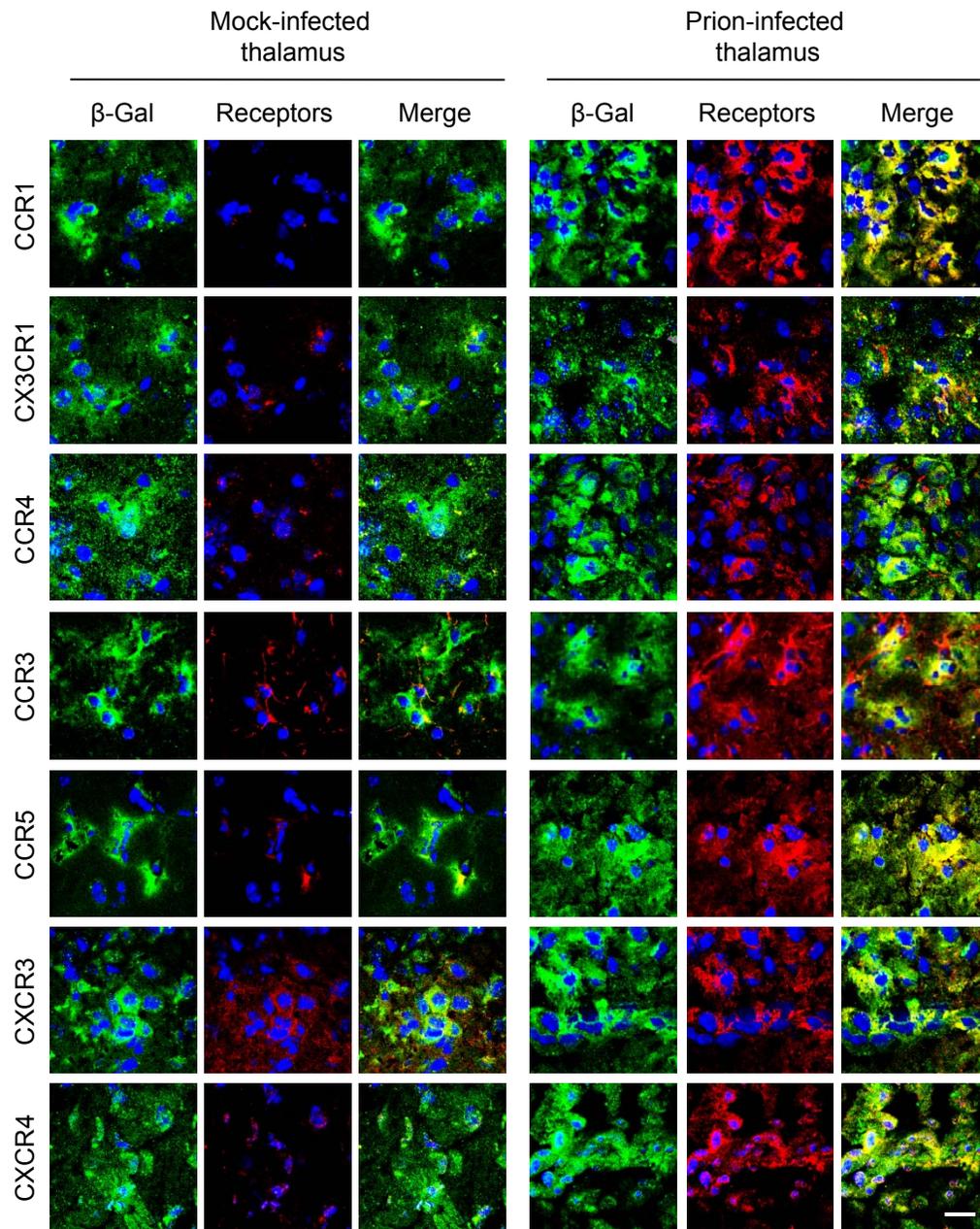


Fig. 5 Song *et al.*
To be continued

B

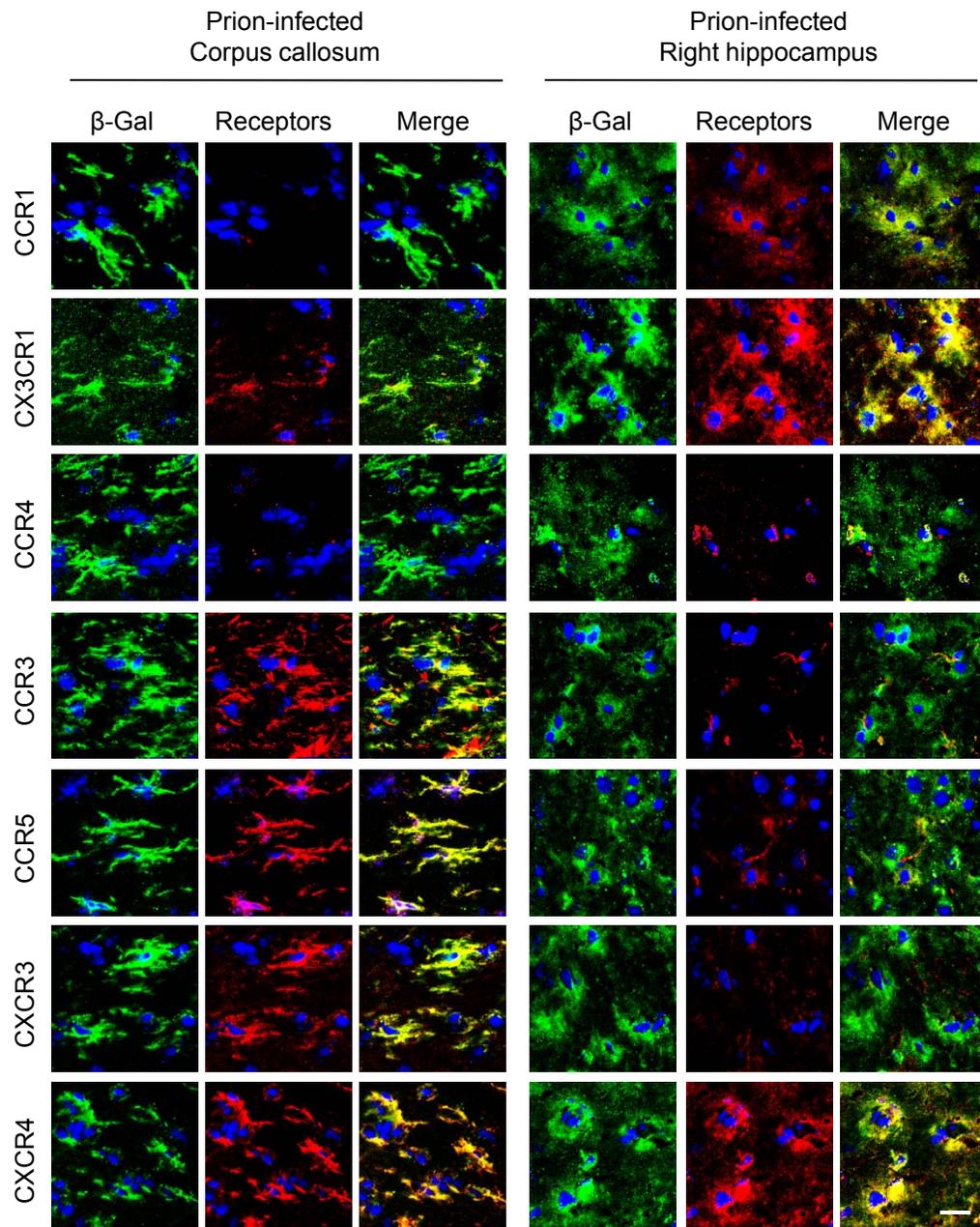


Fig. 5 Song *et al.*