의학석사 학위논문

인간중간엽 줄기세포의 간으로의 이동에 중요한 인자인 케머린에 대한 연구

Chemerin is a critical factor that enhances hMSC homing to the liver.

2019 년 6 월

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Abstract

Chemerin is a critical factor that enhances hMSC homing to the liver.

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Stem cell therapy is a promising therapeutic approach to cure various diseases. However, it is difficult for stem cells to specifically home to damaged tissue. The previous study demonstrated that when human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) were engrafted in mouse liver fibrosis model, it migrated to the liver. This finding was important because most of the hE-MSCs injected in the mouse went to the liver. In this study, I explored the mechanism of hMSC homing to the liver. By

performing cytokine array between normal and 1day thioacetamide (TAA) treated mouse liver and I found that Chemerin level increased the most. From this result, I treated Chemerin to hE-MSCs and hBM MSCs (Bone Marrow Mesenchymal Stem cell) and found that Chemerin enhanced 2D and 3D migration of these cells. Next, treatment of Chemerin activated phosphorylation of JNK1.2, ERK1/2, p38 in hBM MSCs. Moreover, Chemerin induced mRNA and protein level of CD44, ITGA4 and MMP2 which are involved in transmigration. Furthermore, transcription factors associated with transmigration, MZF1, GATA3, STAT3 and STAT5A were triggered by Chemerin. To validate that Chemerin enhances migration of hBM MSCs, CMV-Flag-Mouse Chemerin vector was made and injected into mice to specifically overexpress in the liver. I discovered that hBM MSCs migrated more in mice that were overexpressed with Chemerin. Together, these results indicate that Chemerin is the molecule that enhances hMSCs to mouse liver.

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Keywords: Chemerin; Migration; Homing; MSC; Liver; CMKLR1

Student number: 2016-28120

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LIST OF ABBREVIATIONS

hE-MSC: Human Embryonic Mesenchymal Stem Cell

hBM MSC: Human Bone Marrow Mesenchymal Stem Cell

CMKLR1: Chemokine-like receptor 1

JNK: c-Jun N-terminal kinase

P38: Mitogen-activated protein kinases

ERK1/2: Extracellular signal-regulated kinase 1/2

TAA: Thioacetamide

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INTRODUCTION

Stem cell therapy is a new rising method that is used to cure various diseases. Because of its capacity to infinitely proliferate, self-renewal and differentiate into many different cell types, stem cell is considered as next regenerative therapy[1]. There are many different types of stem cell but the main two categories are divided into embryonic stem cell and adult stem cell[2]. Embryonic stem cell has the most pluripotent ability to differentiate into many different types of cells, but to use in stem cell therapy there are several ethical issues. Therefore, people considered using adult stem cell, especially, mesenchymal stem cell since it can be easily obtained from human bone marrow, lacks immunogenicity, lacks ethical concerns and can differentiate into specific cell types[3].

Despite the fact that hMSCs have the potential to cure many diseases, stem cell therapy is still uncommonly used. This is because of their low ability to migrate to a specific tissue when transplanted into recipients[4]. Many studies have focused on increasing the homing ratio of MSCs by pre-exposing it to cytokines or incubate in a hypoxia condition which increases the

expression of its receptor[4]. Another method used to increase homing is to genetically engineer MSCs to overexpress cytokine receptors or integrin alpha 4, which help MSCs in transmigration[4].

Chemerin is a small(16kda) protein that is mostly made and secreted by the liver, adipocyte and lung[5]. Once secreted, it circulates in the blood until it gets processed by different proteases depending on the injured site[5]. C terminal of Chemerin acts as a crucial site for enzymatic activity and amino acid cleavage at this site determines the biological activity of Chemerin [6, 7]. Moreover, Chemerin plays a role in adipogenesis, angiogenesis, osteoblastogenesis, myogenesis, and glucose homeostasis[8]. On top of all these roles, Chemerin is also involved in chemotaxis by recruiting immune cells to the injured site [8]. Overall, Chemerin is a multifunctional protein that is used in various tissues depending on the injured sites.

In this study, I found Chemerin as the cytokine that recruits hMSCs to the liver by performing cytokine array between normal and TAA treated mouse liver. I confirmed that Chemerin enhances migration of hE-MSCs and hBM MSCs in vitro and in vivo. Furthermore, I investigated the molecular mechanism of Chemerin in hBM MSCs and found that Chemerin triggers

phosphorylation of JNK1/2, ERK1/2, p38. Also, this protein increases migratory gene levels, CD44, ITGA4 and MMP2 thus enhancing hBM MSCs transmigration. Moreover, transcription factors involved in cell transmigration, MZF1, GATA3, STAT3, STAT5A, get regulated by Chemerin. Lastly, the animal experiment was performed by specifically increasing protein level of Chemerin in mouse liver and confirmed that hBM MSCs migrated more in Chemerin vector injected mouse. Therefore, I concluded that Chemerin is the critical protein that enhances MSC homing to the liver.

MATERIALS AND METHODS

Cell culture

Human embryonic stem cells (hE-MSC) were obtained using a previously reported protocol [9]. hE-MSC were cultured in EGM-2MV medium (Lonza, Switzerland, CC-3202). Bone marrow Mesenchymal Stem cells were bought from LONZA (Lonza, Switzerland, PT-2501) and was cultured in Mesenchymal Stem Cell Growth Medium 2 (Promocell, Germany, C-28009). Hepa1c1c7 cells were cultured in 10% FBS MEM (Thermo Fisher Scientific, United State, 11095080). HUVECs were cultured in EGM 2MV (Lonza, Switzerland, CC-3202). MS-1 cells were cultured in 5% FBS DMEM (Thermo Fisher Scientific, United State, 11995065).

FBS added to DMEM and MEM media is from Gibco (Thermo Fisher Scientific, United State, 16000)

Chemokine array

Proteome Profiler Mouse Chemokine Array Kit (R&D system, ARY020) was according to manufacture protocol.

Western Blot

Cells were harvest in RIPA buffer (Thermo Fisher Scientific, United State, 89900) and were agitate in ice for 15min. After 15minutes these lysates were centrifuged at 13000rpm, 4℃ for 10 minutes. Then the supernatants were collected and quantified using BCA protein assay kit (Thermo Fisher Scientific, United State, 23225). Proteins were loaded in 10% Tris-glycine SDS-polyacrylamide gel and transferred. Once the transfer was done, membranes were blocked in 5% Normal Horse Serum(NHS) (Sigma-Aldrich, United State, H1270-500ML) for 1hour and afterwards primary antibodies were added and incubated overnight:

Quantitative PCR

RNA was isolated from cells using TRIzol (Invitrogen, United State, 15596026) using manufactures protocol. Then RNA concentration was quantified with Nanodrop and 1ug RNA was transcribed into cDNA using RT Master Premix(oligo d(T) kit (Elpis Biotech, Korea, EBT-1512). Quantitative PCR was performed using FastStart Universal SYBR Green Master (Rox) (Merk Millipore, Germany, 4913850001)

Primer Sequence

hCD44 Forward: 5’ CGAAGAAGGTGTGGGCAGAAG 3’
hCD44 Reverse: 5’ CGACTCCTTGTTCACCAAATGC 3’
hITGA4 Forward: 5’ TAGCCCTAATGGAGAACCTTGTG 3’
hITGA4 Reverse: 5’ TCTATGCCCACAAGTCACGATG 3’
hMMP2 Forward: 5’ GCCAAGTGGTCCGTGTGAAG 3’
hMMP2 Reverse: 5’ CAAAGTTGTAGGTGGTGGAGCA 3’
hGATA2 Forward: 5’ GCCACAGCCACCCCTCTC 3’
hGATA2 Reverse: 5’ GGTTGTCGTCAGTCTTCGCTT 3’
hGATA3 Forward: 5’ CACCCCATCACCACCTACCC 3’
hGATA3 Reverse: 5’ CCTGCCTGTGCTGGACCG 3’
MZF1 Forward: 5’ CTTCTCCCCAGGGTTCGC3’
MZF1 Reverse: 5’ GCGGGAGGGTGATTGGAT 3’
hSTAT3 Forward: 5’ CTAGAGACCCACTCCTTGCCAG 3’
hSTAT3 Reverse: 5’ TTTACATTCTTGGGATTGTTGGT 3’
hSTAT5A Forward: 5’ GTCCTGAAGACCCAGACCAAGT 3’
hSTAT5A Reverse: 5’ CTCGTTGCGGGTGTTCTCAT 3’

2D migration

$1.0x10^4$ hBM MSCs and $2.5x10^4$ hE-MSCs were plated in each of the ibidi culture-insert 2 Well (ibidi, United State 81176) and were incubated in CO^2 overnight. Next day, insert 2 well was removed and exchanged medium to 0.5% FBS. Then 50 pM of mChemerin (R&D System, United State, 2325-CM-025) was added to cells. Cells were observed every 6hrs.

3D migration

Hepa1c1c7 cells transfected with Plenti-CMV-Flag- mouse Chemerin were trypsinized and mixed with collagen from 3D collagen cell culture system (Merk Millipore, United State, ECM 675). 300 ul of the collagen-cell mixture were plated in 8 well (ibidi, United State 80826) and was solidified in 5% CO^2 incubator for 1hour. After solidification of collagen, $5x10^4$ DiO (Thermo Fisher Scientific, United State, V22886) labelled MS-1 cells were plated. Once MS-1 cells were attached, $5x10^4$ DiI (Thermo Fisher Scientific, United State, C7001) labelled hBM MSCs were plated. These cells were incubated for 2 days and the observed using a confocal microscope (Leica Microsystem, South Korea, Leica STED CW). For hE-MSCs' 3D migration, the same protocol was used above except Hepa1c1c7 cells were not included in collagen mix.

Vector

Chemerin originally have a signalling peptide at the N-term and gets enzymatic cleave at the C-terminal. Therefore, we designed a plenti-CMV-Flag-mChemerin vector as an active form that does not have the last 6 amino acid and flag was tagged at the

N-terminal. We used pLenti-C-mGFP (origene) as a vector and then ligated mChemerin sequence into the vector.

Animal experiment.

Mouse Liver Fibrosis Model and Cell Transplantation

BALB/c-nude mice (male, 12-13 weeks old, 20-25 g) were used for all animal experiments. Mice were administered with TAA 200 mg/kg (Sigma-Aldrich, United State, 163678) by intraperitoneal injection three times per week for 14 days. The negative control group was injected with 0.9% saline. Cells were transplanted by intracardiac injection 1 day after the first TAA injection (Fig. 1A). Before cell injection, hE MSCs were stained with 4 mg/mL CellTracker CM-DiI (Thermo Fisher Scientific, United State, C7000) at 37℃ for 24 hr. At 14 days after cell transplantation, liver tissue was harvested for histology.

CMV-Flag-mChemerin vector Injection

CMV-Flag-mChcmeirn vector and emerald GFP vector were incubated with by lipotrust-vitamin A complex. Lipotrust(CSR-LEO-10-EX, Cosmobio):Retinol(R7632, Sigma) were mixed in ratio of 240nmol:120nmol. Then 18.5ug vector was added to

100nmol of Retinol:lipotrust complex and incubated for 20 minutes at room temperature. After 20 minutes, I filtered this complex with PES column at 1500g for 5 minutes, 3 times at 25℃. Then this complex was eluted with PBS to make a total volume of 100ul. I injected these vector complex into BALB/c-nude mouse through intraperitoneal injection. Two days after vector injection, hBM MSCs were injected through cardiac injection. For hBM MSC injection, $1.0x10^6$ hBM MSCs were collected and incubated with CM-DiI Dye (1mg/ml) (Thermo Fisher Scientific, United State, C7000) for 5 minutes in 37C and then incubated in 4℃ for 10minutes. After 15 minutes, centrifuged the cells at 1200rpm for 5 minutes and wash with PBS 2 times. Then cells were suspended in PBS and $1.0x10^5$ were injected into each mouse (Fig. 4G). Two days after hBM MSC injection (5days after vector injection), mouse liver were harvest and were fixed in 4% paraformaldehyde for 3 and 7 days or freeze in LN2 tank for protein analysis.

Immunofluorescence

Tissue harvested from mouse were fixed in 4% paraformaldehyde for 3 days and exchanged in 15% sucrose, 30% sucrose gradually until the tissue sank. Then tissue block was made by embedding it in optimal cutting temperature compound (OCT) in the chilling condition below −20℃ degree. Next, these tissues were cryosection at 7uM. DAPI was stained for 15mintues in PBS and washed with PBS 3 times. DiI hBM MSCs were overserved in each group with 555nM laser using Leica confocal microscope.

Statistical Analysis

Experiments and groups were triplicates, and all data were calculated as mean ± SD. Group comparisons were performed by T−test, and a number of asterisks on top of each graph mean statistical significance, “*”,”**”,”***” means that p−value range is 0.01 to 0.05, 0.001 to 0.01, 0.001 to 0.01, respectively.

RESULT

Screening of chemoattractant that recruits hE-MSC in mouse injury model.

In the previous study, I constructed a mouse liver fibrosis model using a well-established thioacetamide (TAA) induced liver fibrosis protocol. Then, hE-MSCs were injected according to the time table presented in Fig. 1 to confirm the therapeutic effect of hE-MSC. In order to visualize the cell, hE-MSCs were labelled with DiI. 14 days after cell injection, livers were harvested and hE-MSCs were observed (Fig. 1A). Next, I thought that hE-MSC homing would be different between normal and fibrotic liver since distinct cytokines are secreted from these two livers. Therefore, chemokine array was performed between normal and 1day TAA treated mouse liver. Short exposure of the film showed an increased level of Chemerin in TAA treated mouse than the normal liver. Moreover, longer exposure showed an increased level of CXCL2 and CXCL10 in TAA treated mouse (Fig. 1B). These results suggest that either Chemerin, CXCL2 or CXCL10 recruit hE-MSCs to the liver.

Chemerin recruits hE-MSC and hBM MSC in vitro

The previous result showed increased levels of Chemerin, CXCL2 and CXCL10 in TAA treated liver. I thought that these molecules might enhance hE-MSC migration to the liver. Therefore, 2D migration of hE-MSCs was performed by adding 50 pM of CXCL2, CXCL10 and Chemerin to each dish. Migration of hE-MSCs was taken pictures at 0hour, 12hour and 20hours. At 12 and 20 hours, hE-MSCs treated with Chemerin migrated the most (Fig. 2A). Moreover, to mimic in vivo environment, 3D migration was performed by seeding DiO labelled HUVECs and DiI labelled hE-MSCs on top of collagen that were added with chemokines (CXCL2, CXCL10 and Chemerin). After 72hours, hE-MSCs plated on top of Chemerin added collagen, migrated the most (Fig. 2C).

Nowadays, hBM MSCs are more widely applicable than hE-MSCs so I wondered if hBM MSCs could also migrate due to Chemerin. It is known that G protein-coupled receptor, CMKLR1, is a receptor for Chemerin in cell migration[10]. Therefore, protein expression of CMKLR1 in hBM MSCs was observed using westernblot(Fig. 2D). To test whether hBM MSCs could migrate due to Chemerin, 2D migration was performed. Similar to hE-

MSCs, hBM MSCs migrated more in Chemerin treated dish (Fig. 2 E, F). Moreover, since hBM MSCs migrate due to Chemerin and CMKLR1 interaction, I predicted that by blocking CMKLR1 in hBM MSCs cell migration would hinder. Hence, 15 minutes prior to Chemerin addition, CMKLR1 was neutralized with antibody and 2D migration was observed. As predicted, even though the same amount of Chemerin was added to each dish, compare to naïve hBM MSC, CMKLR1 blocked hBM MSC migration slowed down (Fig. 2E, F). Therefore, I concluded that Chemerin enhances hBM MSC migration and by blocking Chemerin receptor, CMKLR1, hBM MSC migration decreased.

Chemerin triggers phosphorylation of JNK1/2, ERK1/2 and p38 and increases migration related gene expression in hBM MSC

Next, I investigated the molecular mechanism of Chemerin on hBM MSCs. It has been reported that when Chemerin interacts with CMKLR1, it triggers phosphorylation of JNK1/2, ERK1/2 and p38[11]. Therefore, hBM MSCs were treated with Chemerin and detected protein levels after 30 minutes. All of these proteins'

phosphorylation levels were increased due to Chemerin (Fig. 3A). From these data, I suggest that JNK1/2, ERK1/2 and p38 are upregulated by Chemerin.

Since Chemerin is a chemotactic protein that enhances cell migration, I searched for transmigration molecules that would help hBM MSCs transmigrate between endothelial cells to a specific tissue. I selected CD44, VLA4 and MMP2 because these proteins were well known to be involved in the transmigration of hBM MSCs [12,4]. Hence, Chemerin was treated to hBM MSC for 24hours and obtained mRNA and protein levels of CD44, VLA4 and MMP2 have increased (Fig. 3 B, C).

To investigate further, I searched for transcription factors that are high signals of CD44, ITGA4 and MMP2 and involved in cell transmigration. From numerous candidates, GATA2, GATA3, MZF1, STAT3 and STAT5A were selected as the transcription factor that would be regulated by Chemerin (Fig. 4D)[13,14,15,16,17]. I screened these molecules by treating hBM MSCs with Chemerin. After 24hours of treatment, mRNA expression of GATA3, MZF1, STAT3 and STAT5A have increased and expression of GATA2 has decreased (Fig 4E). Protein levels needed to be checked but overall I concluded that GATA3, MZF1, STAT3 and STAT5A

are regulated by Chemerin in hBM MSCs.

Overexpression of Chemerin in mouse liver increased hBM MSC migration

From previous data, I observed that Chemerin enhances hBM MSC migration by increasing migratory protein expression. Therefore, to test the Chemerin effect in vivo, I designed CMV-Flag-Mouse Chemerin vector (CMV-mChemerin) (Fig. 4A). Expression of this vector was tested in 293T cells and media (Fig. 5B). Using the media of CMV-mChemerin transfected 293T cells, I performed 2D migration of hBM MSCs. At 6 and 12 hours, hBM MSCs incubated in media of transfected CMV-mChemerin vector have migrated more than the control group (Fig. 5C). To prove further, 3D migration of hBM MSCs were performed using CMV-mChemerin vector. hBM MSCs seeded on top of mChemerin transfected Hepa1c1c7 collagen mix, migrated more than collagen only set (Fig. 5F). mChemerin expression in Hepa1c1c7 cells was checked by western blot (Fig. 5D). Therefore, 2D and 3D migration showed that overexpression of mChemerin increased hBM MSC migration.

Next, to test whether Chemerin could increase hBM MSC migration in vivo, CMV-mChemerin vector was peritoneally injected into Balc/Nude mouse. Two days after vector injection, DiI labelled hBM MSCs were administered through cardiac injection. 5 days after the vector injection, livers were harvested (Fig. 4 G, Top). Then immunofluorescence was performed and observed that hBM MSCs in CMV-mChemerin vector injected mouse migrated more than the control (GFP vector) mouse (Fig. 5G). Overall, my result indicated that Chemerin enhances hBM MSC migration in vitro and in vivo.

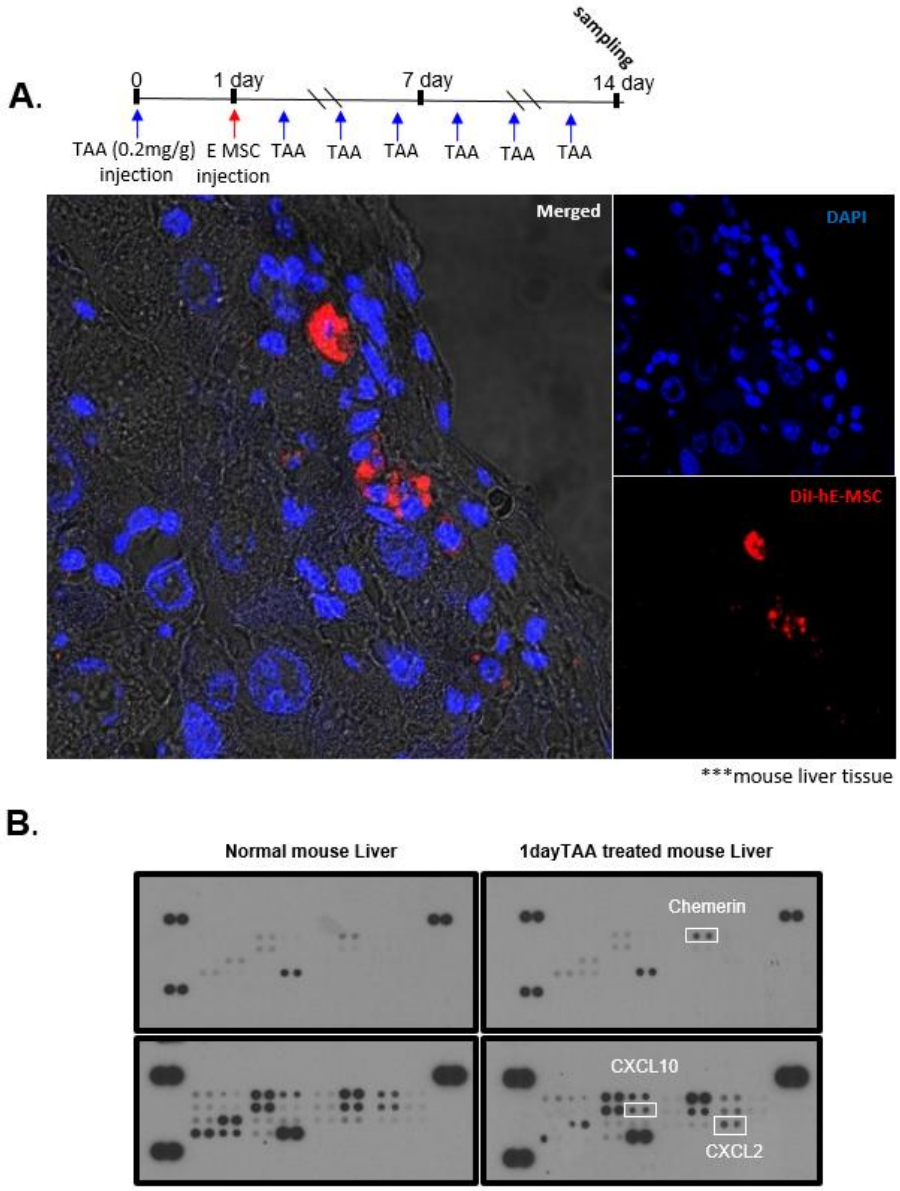
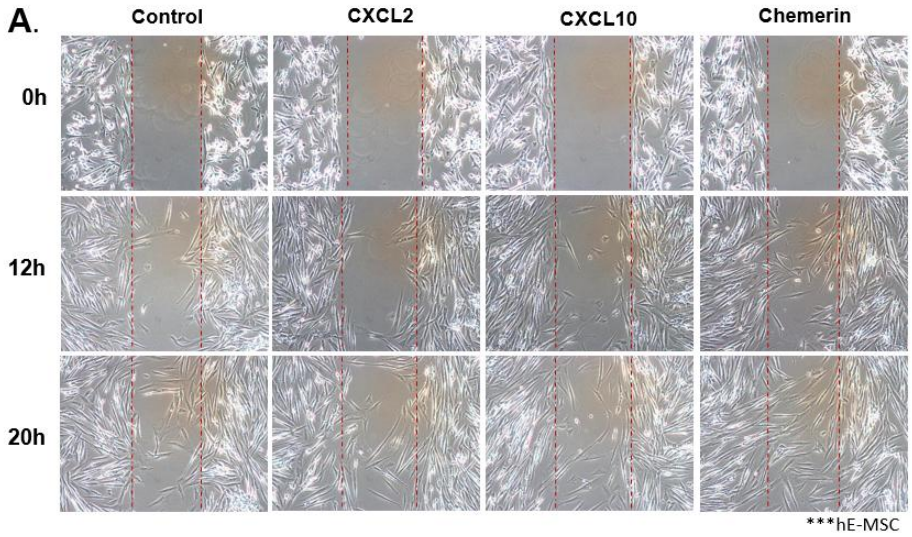


Figure 1. Screening of chemoattractant that recruits hE-MSC in mouse injury model.

A. Immunofluorescence of DiI labelled hE-MSC in mouse liver treated with TAA.

B. Mouse Chemokine array blots of normal and 1day TAA treated mouse liver. The top part was exposed 5 minutes and the bottom part was exposed 10minutes.



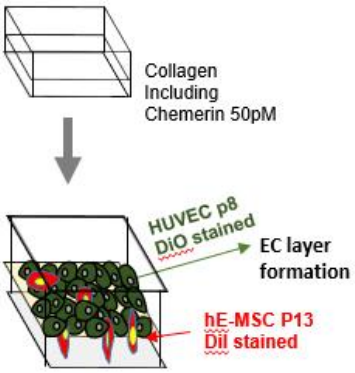
B. **Scheme of 3D transmigration assay**

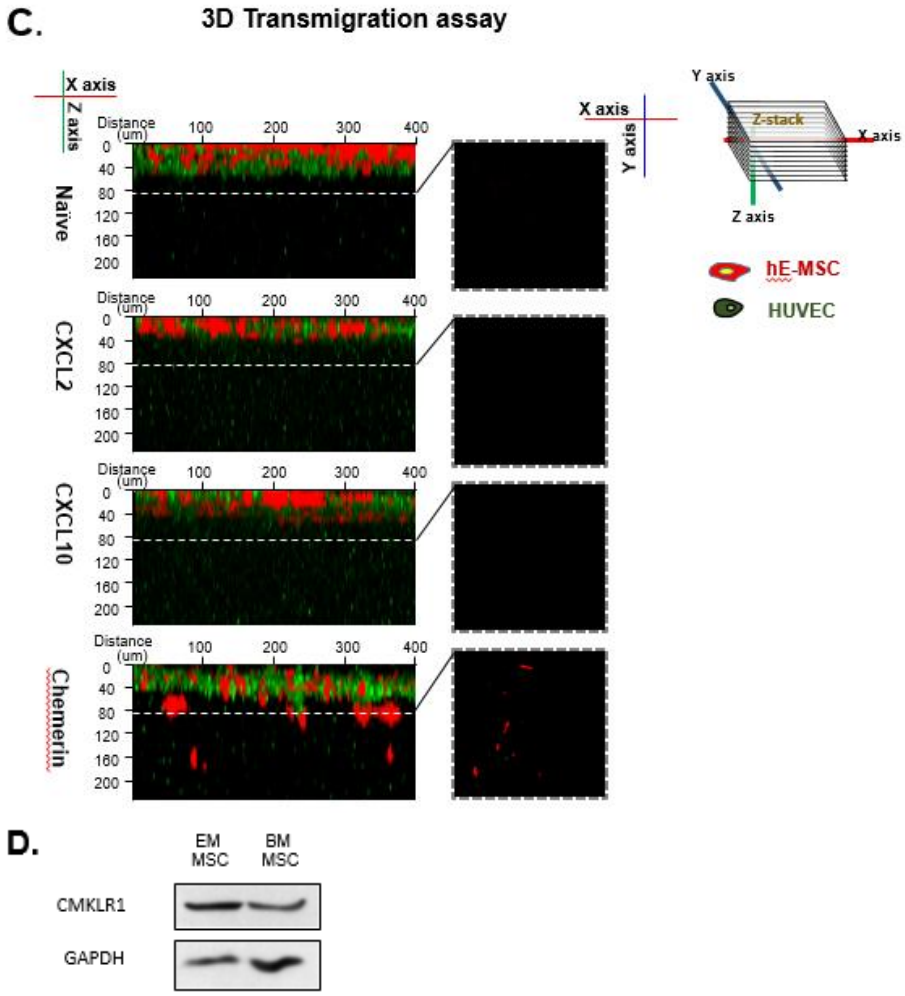
1. Gel solidify including 0.5% FBS and Chemerin 50pM for 1hr at 37'C

2. HUVEC p8 staining with DiO and plating in EBM including 0.5% FBS and stabilizing for 2hr

3. hE-MSC p13 staining with DiI and plating in EBM including 0.5% FBS and stabilizing for 2hr

4. Incubation for 48hr and 72hr until analysis with confocal




C.
3D Transmigration assay
X axis
Z axis
Distance (um)
100
200
300
400
0
40
80
120
160
200
Naive
CXCL2
CXCL10
Chemerin
X axis
Y axis
Y axis
Z-stack
X axis
Z axis
hE-MSC
HUVEC
D.
EM MSC
BM MSC
CMKLR1
GAPDH

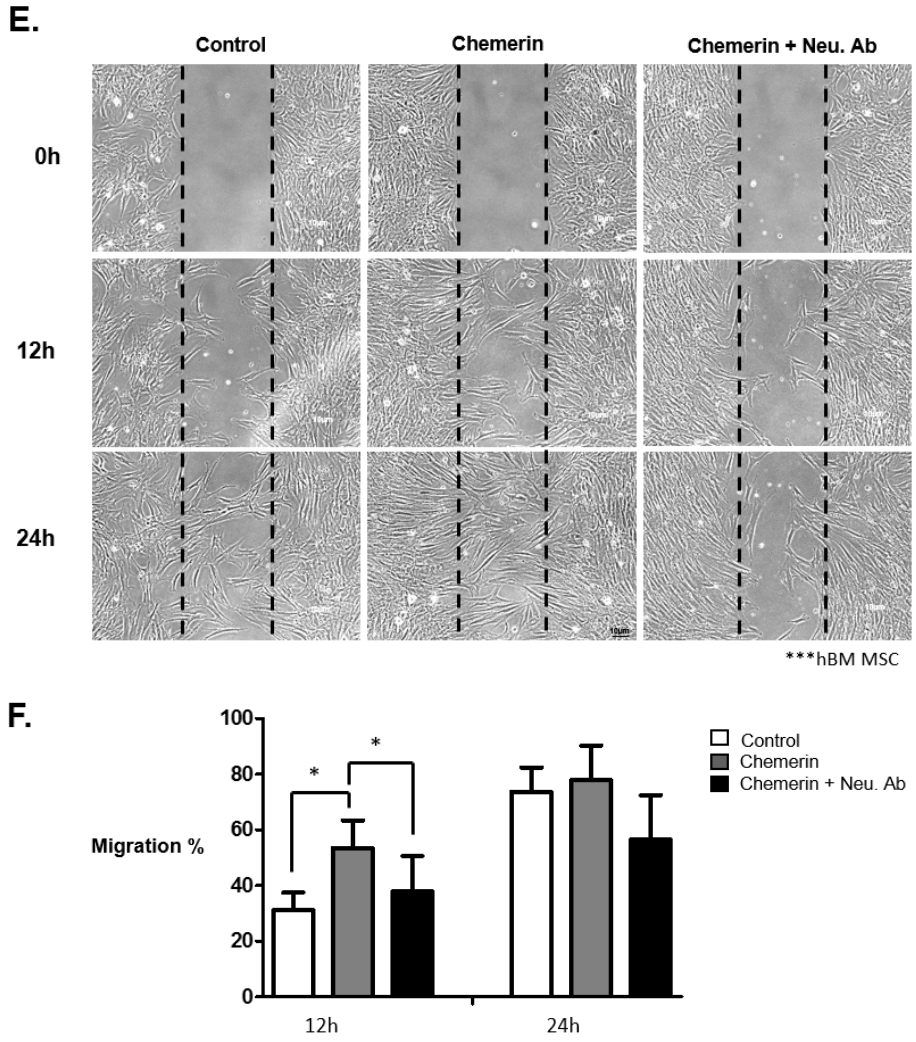


Figure 2. Mouse chemerin recruits hE-MSC and hBM MSC in vitro

A. 2D migration of hE-MSCs that are exposed to Chemerin (50pM), CXCL2 (50pM) and CXCL10 (50pM). Migration pictures were taken at 0h, 3h, 6h, 12h, 20h.

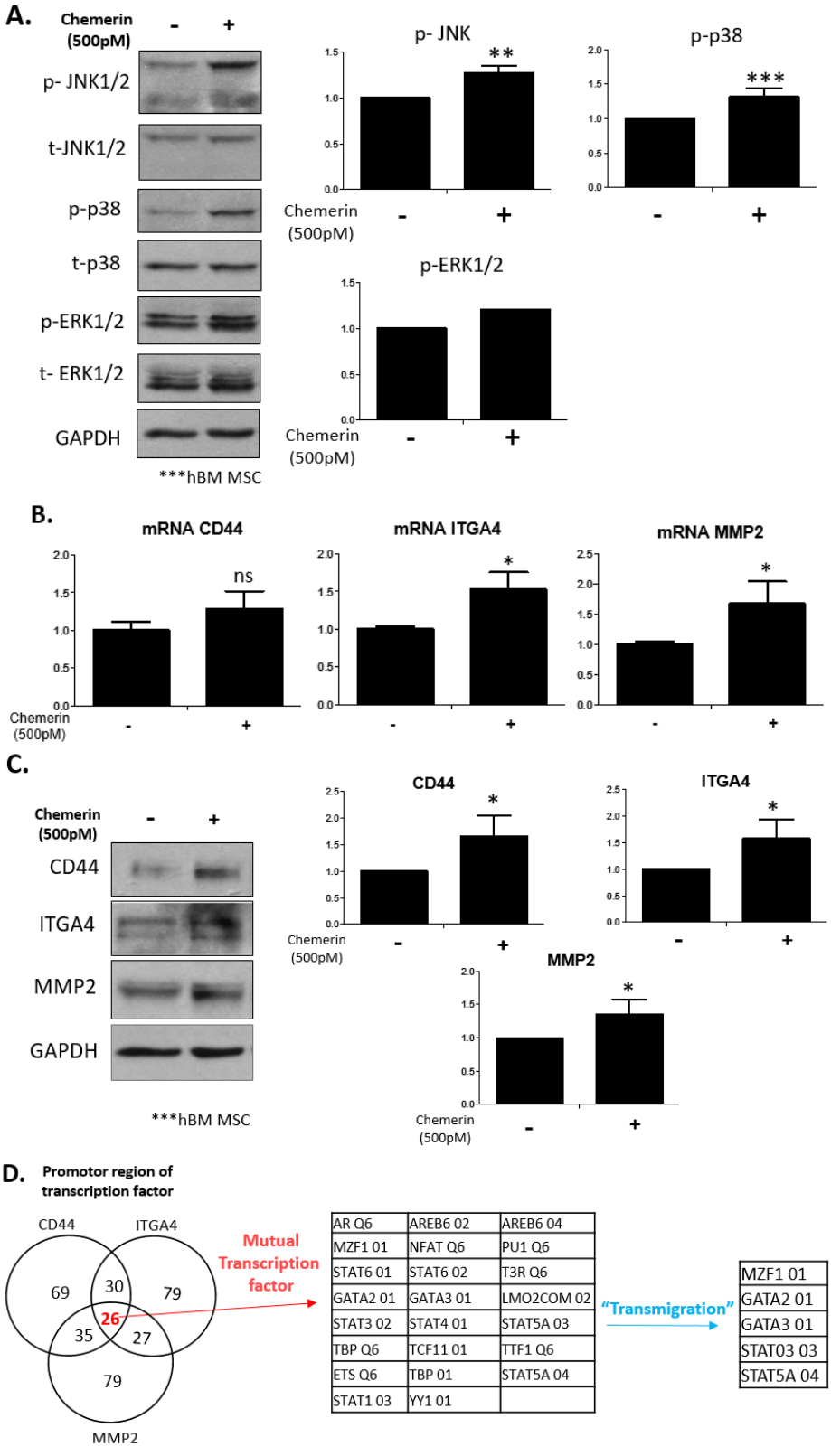
B. Scheme of 3D transmigration assay.

C. 3D transmigration of hE-MSCs using HUVECs as an endothelial layer. hE-MSC were treated with CXCL2, CXCL10, Chemerin (all treated at 50 pM). HUVECs labelled with DiO (green) and hE-MSCs labelled with DiI(red).

D. The CMKLR1 protein level in hE-MSCs and hBM MSCs.

E. 2D migration assay of hBM MSCs in response to Chemerin and CMKLR1 blocker (CMKLR1 neutralizing antibody).

F. Quantitative graph of 2D migration of hBM-MSCs. Cell migration was calculated and expressed as the percentage of cell coverage to the initial cell-free zone.



AR Q6	AREB6 02	AREB6 04
MZF1 01	NFAT Q6	PU1 Q6
STAT6 01	STAT6 02	T3R Q6
GATA2 01	GATA3 01	LMO2COM 02
STAT3 02	STAT4 01	STAT5A 03
TBP Q6	TCF11 01	TTF1 Q6
ETS Q6	TBP 01	STAT5A 04
STAT1 03	YY1 01	

MZF1 01
GATA2 01
GATA3 01
STAT03 03
STAT5A 04

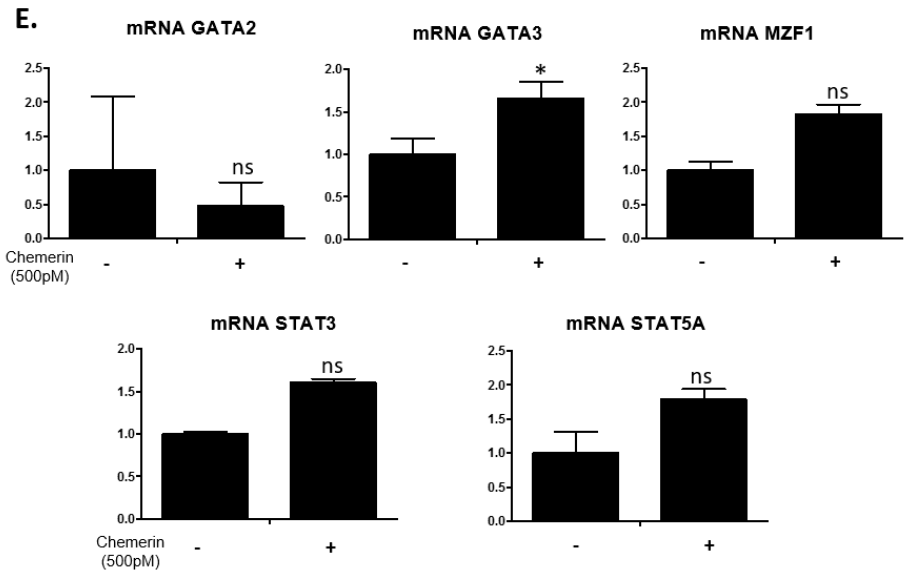


Figure 3. Chemerin triggers phosphorylation of JNK1/2, ERK1/2and p38 and increases migration related gene expression in hBM MSC

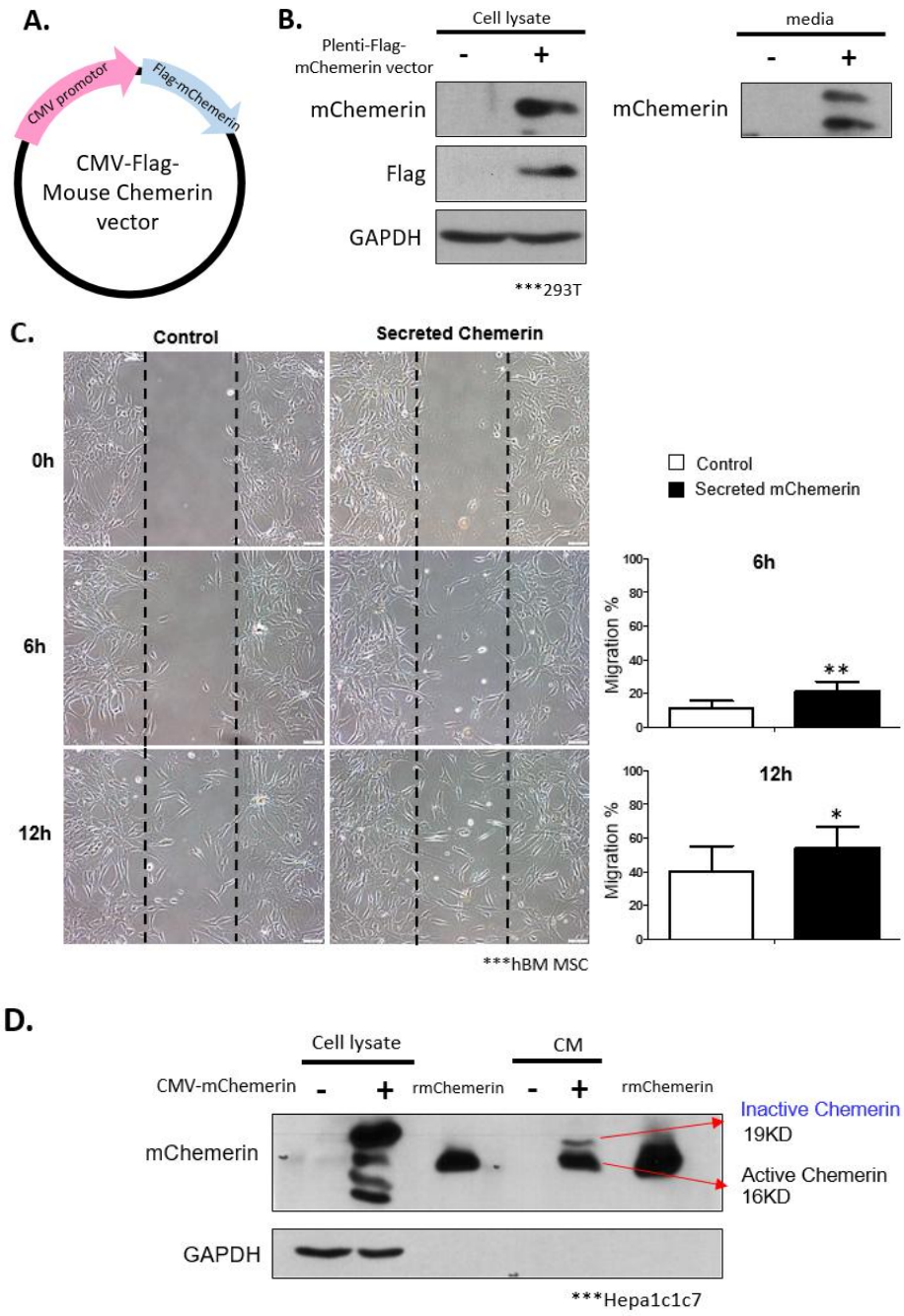
A. JNK1/2, p38 and ERK1/2 phosphorylation were analyzed by western blot when treated with Chemerin. On the right, the protein expression level of each kinase was quantified and normalized to total form.

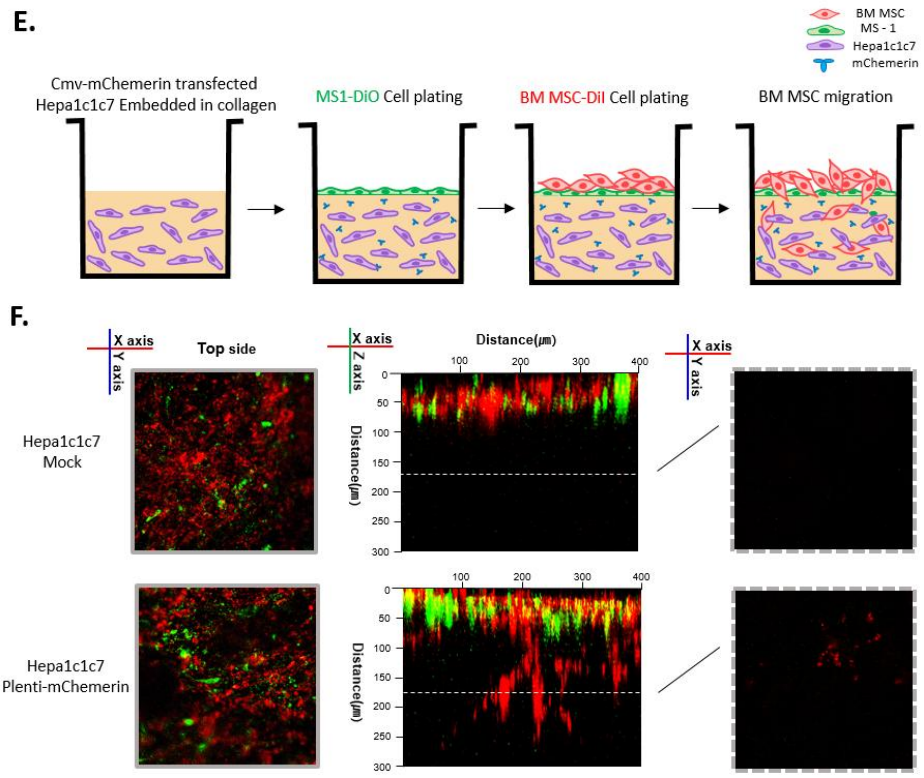
B. mRNA expression of CD44, ITGA4 and MMP2 in the presence of Chemerin.

C. The protein level of CD44, ITGA4 and MMP2 increased when treated with Chemerin (500pM) On the right, protein levels of migratory genes were quantified and normalized to GAPDH.

D. Van diagram of mutual transcription factors of CD44, ITGA4 and MMP2.

E. mRNA level of transcription factors was analyzed in the treatment of Chemerin(500pM)


A.
CMV promotor
Flag-mChemerin
CMV-Flag-
Mouse Chemerin
vector
B.
Cell lysate
media
Plenti-Flag-
mChemerin vector
-
+
mChemerin
Flag
GAPDH
***293T
C.
Control
Secreted Chemerin
0h
6h
12h
Control
Secreted mChemerin
6h
12h
Migration %
**
*
***hBM MSC
D.
Cell lysate
CM
CMV-mChemerin
rmChemerin
mChemerin
GAPDH
Inactive Chemerin
19KD
Active Chemerin
16KD
***Hepa1c1c7


E.
BM MSC
MS - 1
Hepa1c1c7
mChemerin
Cmv-mChemerin transfected
Hepa1c1c7 Embedded in collagen
MS1-DiO Cell plating
BM MSC-DiI Cell plating
BM MSC migration
F.
X axis
Y axis
Top side
X axis
Z axis
Distance(㎛)
X axis
Y axis
Hepa1c1c7
Mock
Hepa1c1c7
Plenti-mChemerin

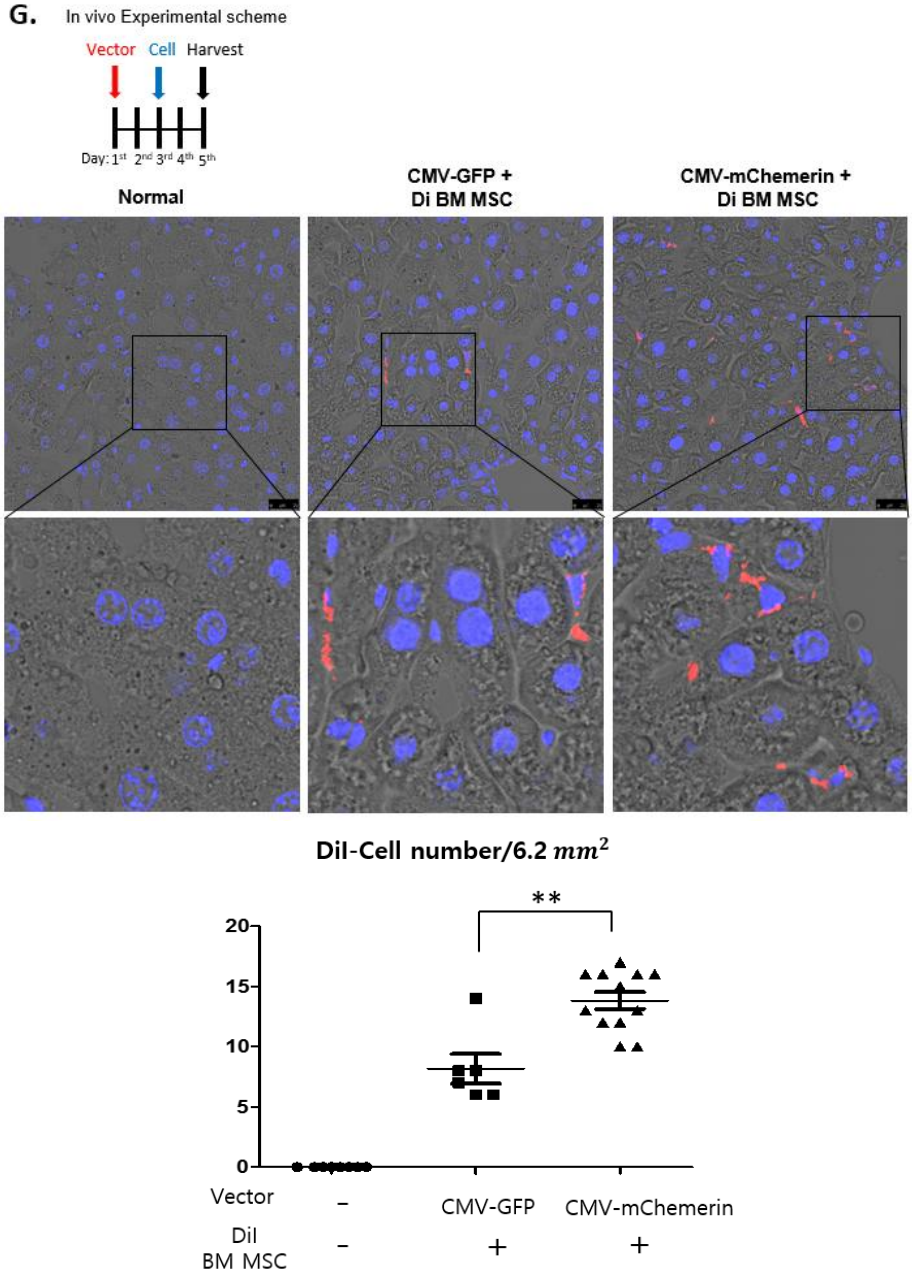


Figure 4. Overexpression of mChemerin in mouse liver, increases hBM MSC migration

A. Vector diagram of CMV-Flag-mChemerin.

B. The protein level of mChemerin in lysate and media of 293T cell transfected with CMV-mChemerin vector.

C. 2D migration of hBM MSCs in mChemerin vector transfected 293T cell medium.

D. The protein level of mChemerin in lysate and media of Hepa1c1c7 transfected with CMV-mChemerin vector.

E. Schematic diagram of hBM MSC 3D migration in CMV-mChemerin transfected Hepa1c1c7 collagen mix.

F. 3D migration of hBM MSCs in CMV-mChemerin transfected Hepa1c1c7 collagen mix. HUVECs labelled with DiO (green) and hBM MSCs labelled with DiI(red).

G. Immunofluorescence of DiI labelled hBM MSCs in animal experiment. Top left shows in vivo experiment scheme. The middle is an image of hBM MSCs in CMV-GFP and CMV-mChemerin vector injected mouse liver. The bottom is a quantitative graph of hBM MSCs in each liver.

DISCUSSION

Stem cell therapy has been one of the recent technology to treat many diseases. However, its ability specifically migrate to a diseased tissue has been a problem. In the previous study, when engrafted hE-MSCs to the mouse that has liver fibrosis, I discovered that most hE-MSCs went to the liver. Therefore, in this study, I searched for the mechanism behind this migration. Firstly, I wondered what caused hE-MSCs to migrate mostly to the liver. Hence, cytokine array was performed between normal and fibrosis liver and found that Chemerin, CXCL2 and CXCL10 levels were increased in TAA treated mouse. According to this result, I performed 2D and 3D migration of hE-MSCs using these 3 cytokines and obtained that in Chemerin treated cells have migrated the most compared to CXCL2 and CXCL10. Therefore, I concluded that Chemerin enhances hE- MSC migration the most.

Next, I tested whether Chemerin could intensify hBM MSC migration as well. Firstly, I checked the expression of Chemerin receptor, CMKLR1 (Chemokine-like receptor 1), which is known to be involved in cell migration [10]. Then, 2D migration of hBM MSCs performed by blocking CMKLR1 receptor with

neutralizing antibody and then treated Chemerin. At 12 and 24hours, hBM MSCs treated with Chemerin have migrated the most. On the other hand, when CMKLR1 is blocked, migration of BM MSCs slowed down even in the presence of Chemerin. These result indicated that Chemerin increased migration of hBM MSCs and by blocking its receptor, cell migration slowed down.

Next, to find out signals that response to Chemerin in hBM MSCs, I searched for references and found that JNK1/2, ERK1/2 and p38 were activated by Chemerin during migration[11]. Hence, Chemerin was added to hBM MSCs and phosphorylation of these proteins was analyzed through western blot. Indeed, phosphorylation of JNK1/2, ERK1/2, and p38 have increased by Chemerin treatment.

In order for MSCs to transmigrate from blood to a specific organ, it needs to go through several steps. The first step of transmigration is that circulating MSC tethers to endothelial cells through CD44 and selectin interaction[4]. Next, VLA4 of MSC interact with VCAM1 of endothelial cells and causes rolling and arrest of MSC[4, 12]. Finally, secretion of MMP2 is required for invasion of the basement membrane of endothelium and cleavage of extracellular matrix to get to desired target[4, 12]. Therefore, I

elected these genes as a migratory gene that would increase by Chemerin in hBM MSCs. In order to see if these genes increase due to Chemerin, hBM MSCs were treated with Chemerin and analyzed CD44, ITGA4 and MMP2 levels after 24 hours. Indeed, all protein and mRNA levels were increased. From these data, I concluded that Chemerin regulates CD44, ITGA4 and MMP2 in hBM MSCs by increasing its expression. Up to this point, I concluded that CD44, ITGA4 and VLA4 are regulated by Chemerin. Furthermore, I know that JNK1/2, ERK1/2 and p38 are down signalling of Chemerin and these signalling proteins might enhance CD44, ITGA4 and VLA4 expression. However, to prove this, a further experiment is needed.

To investigate further, I searched for transcription factors that are regulated by Chemerin and enhances CD44, ITGA4 and MMP2 during cell migration. I used GP Miner(Gene Promoter Miner) to find mutual transcription factors and filtered each gene that was related to transmigration by searching research papers in NCBI (National Center for Biotechnology Information). Among these genes, we choose GATA2, GATA3, MZF1, STAT3 and STAT5A as migration gene that would be regulated by Chemerin[13,14,15,16, 17]. Therefore, by treating Chemerin to hBM

MSC, mRNA level of GATA3, MZF1, STAT3, STAT5A have increased but not MZF1. I am planning to identify if these transcription factors regulate CD44, ITGA4 and MMP2 through Chemerin for future study. From this result, I conclude that Chemerin regulates GATA3, MZF1, STAT3 and STAT5A in hBM MSCs.

Finally, to prove if Chemerin really enhances hBM MSC migrations, I constructed CMV-Flag-mChemerin. Chemerin gets activated by cleavage of last few amino acid at the C terminal depending on the enzyme. Hence, I designed this vector as an active form of Chemerin that do not have the last 6 amino acid, thus can directly bind to CMKLR1 without any processing. To confirm the function of this vector, 293T cells were transfected and used the media for 2D migration of hBM MSCs. The media of CMV-mChemerin transfected 293T cells enhanced the migration of hBM MSCs than the control group. Moreover, to validate this vector further, 3D migration was performed by mimicking space of disse of the liver. I created this environment by embedding CMV-Flag-mChemerin vector transfected Hepa1c1c7 cells (mouse hepatocyte) in collagen which represents the liver. Then DiO labelled MS-1 cells (mouse pancreatic islet endothelial cell)

were added on top of this cell-collagen mix as an endothelial layer of the blood. Finally, DiI stained hBM MSCs were plated on top of these cells and cell migration was observed. Similar to the previous result, I found that hBM MSCs in Hepa1c17 cell transfected with CMV-mChemerin group, migrated the most.

After validating this vector in vitro, I performed an animal experiment to see if Chemerin could increase the migration of hBM MSCs specifically to the liver. I chose liver as hBM MSC homing target because it is wildly known that Chemerin is produced and processed in the liver [18]. I specifically overexpress this vector in the liver by conjugating it with lipotrust-Vitamin A complex since hepatic stellate cells of the liver store 80% of vitamin A in the whole body[19]. Next, this vector was injected into Bal/c Nude mouse by peritoneal injection and 2 days after, cardiac injection of hBM MSC was followed. I observed that hBM MSCs migrated more in the livers that were injected with CMV-mChemerin compared to CMV GFP vector (Fig. 4G). To test whether hBM MSCs can migrate to other organs when specifically overexpressed needs to be studied further.

Overall, I have established a method to increase hBM MSC homing by specifically overexpressing Chemerin to the liver.

Using Chemerin to recruit stem cells could be one of the methods to increase the efficiency of stem cell therapy. However, in order to use Chemerin in stem cell therapy, there are several limitations. When overexpressing Chemerin to a specific organ, it will not only recruit hBM MSCs but also other immune cells and over recruiting immune cells may cause side effect[8]. Therefore, more study needs to be done to use Chemerin in stem cell therapy.

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국문초록

인간중간엽 줄기세포의 간으로의 이동에 중요한 인자인 케머린에 대한 연구

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줄기세포 치료는 다양한 질환을 치료하는데 유망한 치료법이다. 그러나, 줄기세포를 병변조직으로만 호밍(homing)하는데 어려움이 있다. 선행연구에서, 인간 배아줄기세포 유래 중간엽줄기세포 (hE-MSC)를 마우스 간 섬유화 모델에 주입했을 때 이 세포가 간으로 이동함을 규명했다. 이 발견은 마우스에 주입된 대다수의 hE-

MSC 가 간으로 향했다는 점에서 중요하다. 본 연구에서는 무엇이 hE-MSC 를 특히 간으로 이동하게 하는지 주목했다. 따라서, 정상군과 thioacetamide(TAA)를 1 일 처리한 마우스의 간을 사이토카인 분석했을 때, Chemerin 의 수치가 가장 증가하였음을 발견했다. 이러한 결과에서 착안해 마우스 Chemerin 을 hE-MSC 와 hBM MSC 에 처리하였고, 이 세포들이 2D 와 3D 로의 이동이 증가함을 밝혔다. 또한 마우스 Chemerin 이 hBM MSC 에서 JNK, ERK1/2, p38 을 활성화 함을 규명했다. 마우스 Chemerin 의 처리로 이행에 관련 있는 CD44, ITGA4, MMP2 의 mRNA 와 단백질 발현이 증가함을 발견했다. 나아가, 전사인자인 MZF1, GATA3, STAT3, STAT5A 가 마우스 Chemerin 에 의해 촉진됨을 밝혔다. Chemerin 이 hBM MSC 의 이동을 증가시키는지 입증하기 위해 CMV-Flag-mChemerin 벡터를 제작 후, 마우스 간에서 과발현 되도록 주입했다. 그 결과 Chemerin 이 과 발현된 쥐에서 hBM MSC 가 이동되었음을 확인했다. 이와 같은 본 연구의 결과들은 Chemerin 이 hMSC 가 마우스의 간으로 향하게 하는 주요 단백질임을 시사한다.

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주요어: 케머린, 중간엽줄기세포, 간, 이주

학번: 2016-28120